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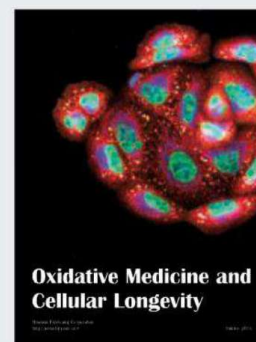
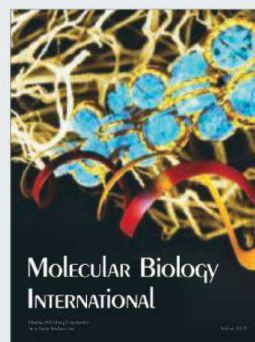
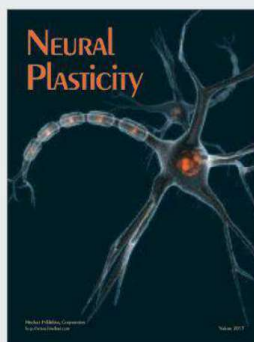
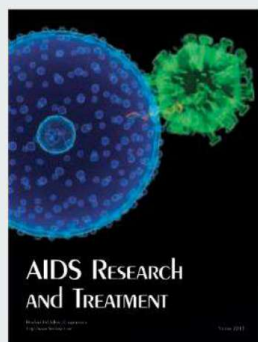
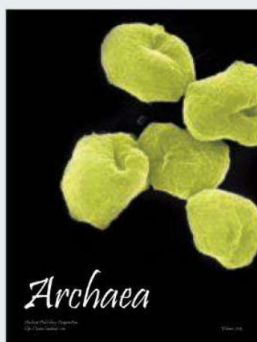
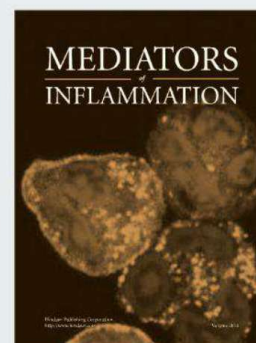
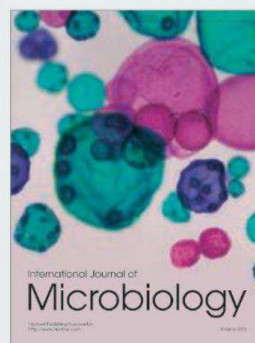
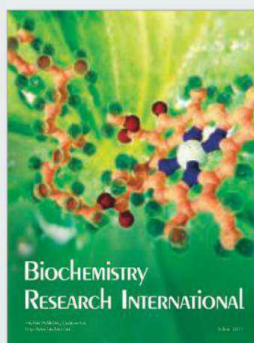
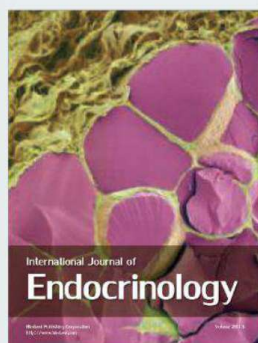
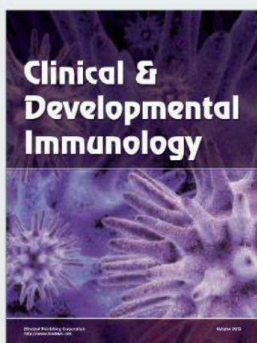
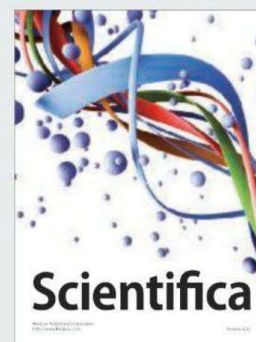
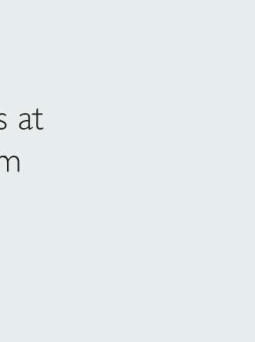
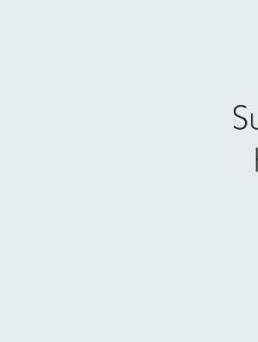
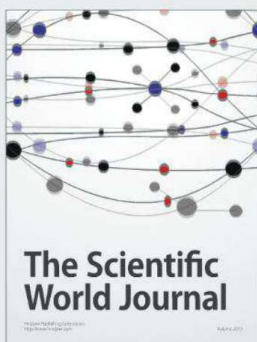
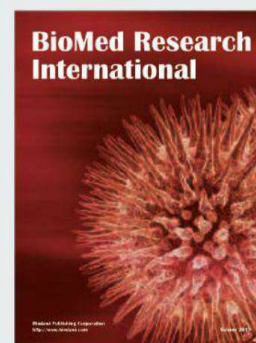
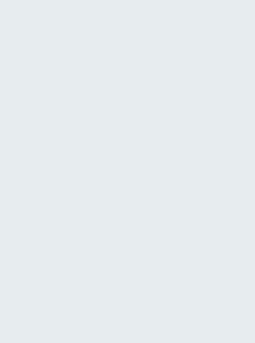
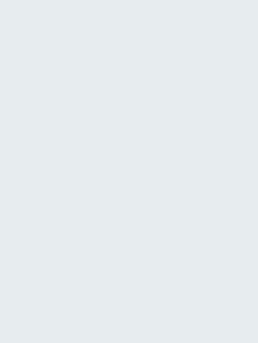
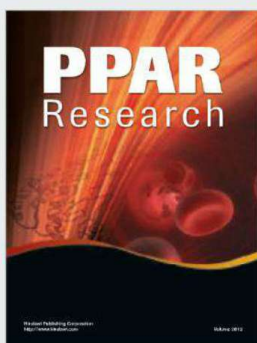
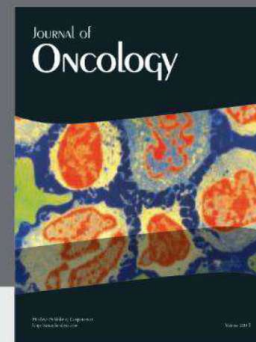
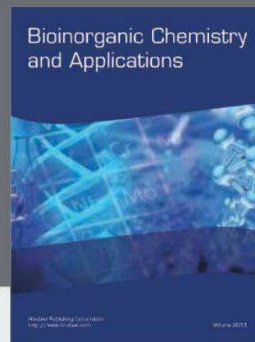
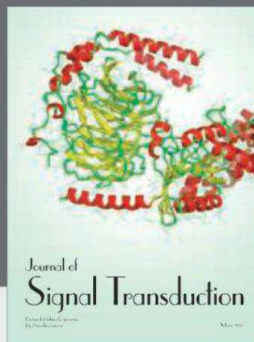
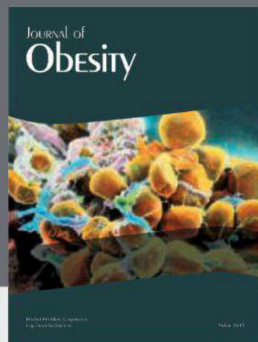
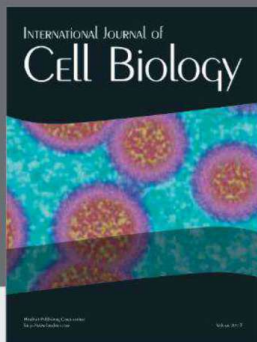
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CRI137B	Recombinant Human IL-15	10 µg
CRI162B	Recombinant Human IL-17	25 µg
CRI172B	Recombinant Human IL-21	10 µg
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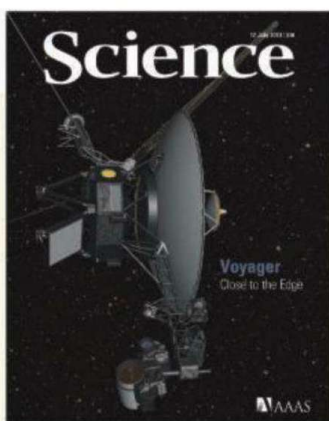
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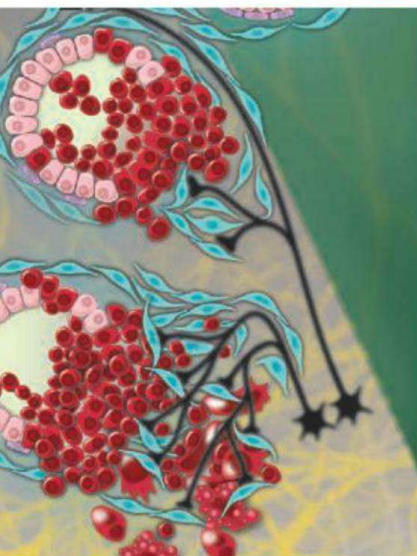
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Rendering of the Voyager 1 spacecraft that left Earth in September 1977. Last summer, when it was 18.5 billion kilometers away and still embedded in the solar magnetic field, Voyager 1 entered an unexpected region, where it observed a sharp decrease of charged particles from the Sun and an abrupt increase in particles from interstellar space. See pages 144, 147, and 150.

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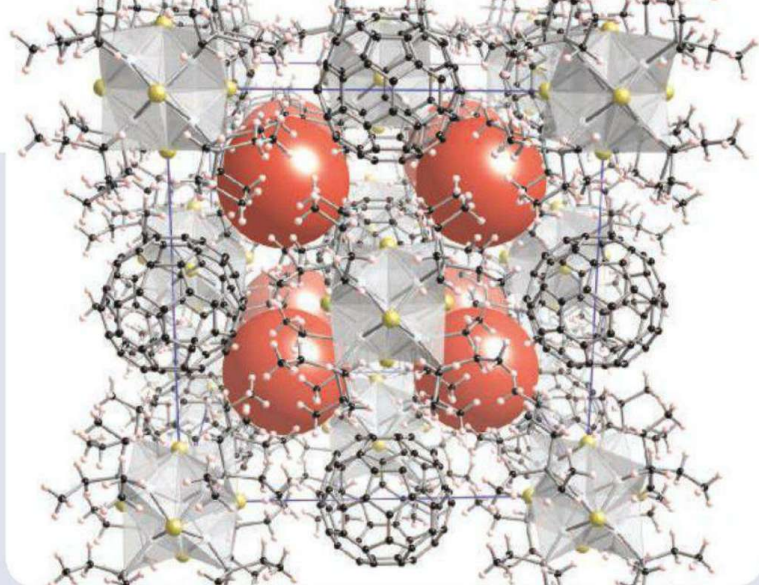
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SCIENCE (ISSN 0036-8075) is published weekly on Friday, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue, NW, Washington, DC 20005. Periodicals Mail postage (publication No. 484460) paid at Washington, DC, and additional mailing offices. Copyright © 2013 by the American Association for the Advancement of Science. The title SCIENCE is a registered trademark of the AAAS. Domestic individual membership and subscription (S1 issues): \$149 (\$74 allocated to subscription). Domestic institutional subscription (S1 issues): \$990; Foreign postage extra: Mexico, Caribbean (surface mail) \$55; other countries (air assist delivery) \$85. First class, airmail, student, and emeritus rates on request. Canadian rates with GST available upon request, GST #1254 88122. Publications Mail Agreement Number 1069624. Printed in the U.S.A.

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Ionic Materials via Charged Clusters

The formation of salts from atomic and small molecular ions could in principle be replicated with larger inorganic clusters. However, many clusters are stabilized by organic ligands that create a barrier for charge transfer reactions to create ions. **Roy *et al.*** (p. 157, published online 6 June; see the Perspective by **Batail**) now report that chromium, cobalt, and nickel selenide and telluride clusters form materials by charge transfer with C_{60} . The Co and Cr clusters formed a layered structure analogous to CdI_2 , while the Ni cluster formed a structure related to $NaCl$.

Unexpected Magnetic Highway

The heliopause is thought to separate the heliosphere (the bubble of plasma and magnetic field originating at the Sun) from interstellar plasma and magnetic field. In August last year, the Voyager 1 spacecraft, which was launched 35 years ago, was 18.5 billion kilometers away from the Sun, close to the expected location of the heliopause. **Krimigis *et al.*** (p. 144, published online 27 June) report observations of energetic ions and electrons by Voyager 1 that suggest that a sharp and distinct boundary was crossed five times over ~ 30 days. **Burlaga *et al.*** (p. 147, published online 27 June) found that the magnetic field direction did not change across any of the boundary crossings, indicating that Voyager 1 had not crossed the heliopause but had entered a region in the heliosphere that serves as a magnetic highway along which low-energy ions from inside stream away and galactic cosmic rays flow in from interstellar space. **Stone *et al.*** (p. 150, published online 27 June) report the spectra of low-energy galactic cosmic rays in this unexpected region.

One-Step Coverage

Controllable formation of thin films often requires slow deposition conditions or multiple rounds of coating. **Ejima *et al.*** (p. 154; see the Perspective by **Bentley and Payne**) report a simple and versatile method for coating surfaces with thin biocompatible films made

from the condensation of Fe^{3+} ions and a natural polyphenol, tannic acid, from aqueous solutions. Flat surfaces, colloidal particles, and even bacterial cells could be coated, and the coats could subsequently be degraded by changing the pH.

From Jawless to Jawed

The earliest vertebrates were jawless. Past reconstructions have assumed that the primitive jawed condition was much like that found in sharks. **Trinajstić *et al.*** (p. 160, published online 13 June; see the Perspective by **Kuratani**) describe fossil musculature from the early jawed placoderms (an extinct class of armored prehistoric fish) that show that the basal structure was distinct from that found in sharks, having a notable dermal joint between the skull and shoulder girdle.

Keeping the Inflammasome in Check

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) play an important role in the detection of pathogens by cells of the innate immune system. For several NLR family members, activation results in relief from autoinhibition, oligomerization, and the recruitment of signaling components that together make up the inflammasome, a large multiprotein complex. The inflammasome protects the host by inducing cell death and cytokine secretion. The specific molecular mechanisms that regulate NLR activation and

inhibition, however, are not well understood. **Hu *et al.*** (p. 172, published online 13 June) report the crystal structure of autoinhibited NLR family member NLRC4, which reveals the domains that are critical for interaction with adenosine diphosphate to keep NLRC4 in its inactive state and the domains that mediate oligomerization of the protein upon activation.

From Nasty to Tasty

Some of our favorite food crops derive from wild relatives that were distasteful or even toxic. Domestication over many years selected for variants with reduced levels of antinutritional compounds. The wild relatives remain valuable, however, for other traits such as resistance to pathogens, but their use in crop development is complicated by the continued presence of unpalatable compounds. **Itkin *et al.*** (p. 175, published online 20 June) elucidate the metabolic pathways and genes directing synthesis of some of these antinutritionals in potato and tomato.

Inward-Facing Antiporter

Calcium/cation antiporters play a role in regulating the cytosolic calcium concentration by using the electrochemical gradient of other cations to catalyze Ca^{2+} transport across cell membranes. The structure of a Na^+/Ca^{2+} exchanger in an outward-facing conformation was recently determined. **Nishizawa *et al.*** (p. 168, published online 23 May) now report the crystal structure of a H^+/Ca^{2+} exchanger in an inward-facing conformation. Comparison of the structures shows how structural changes create hydrophilic cavities to alternate between the intra- and extracellular sides of the protein, facilitating cation transport.



Avian Flu in Ferrets

A recent outbreak of avian H7N9 influenza in humans in eastern China has been closely monitored for any evidence of human-to-human transmission and its potential for sparking a pandemic. **Zhu *et al.*** (p. 183, published online 23 May) examined the behavior of the avian virus in the ferret, a mammalian model for human influenza. The virus was excreted by the ferrets and could be transmitted readily by contact but displayed limited capacity for airborne infectivity. The pathology of H7N9 is similar to H1N1, and it seems that factors other than the intrinsic pathogenicity of the virus contribute to the reported high fatality rate.

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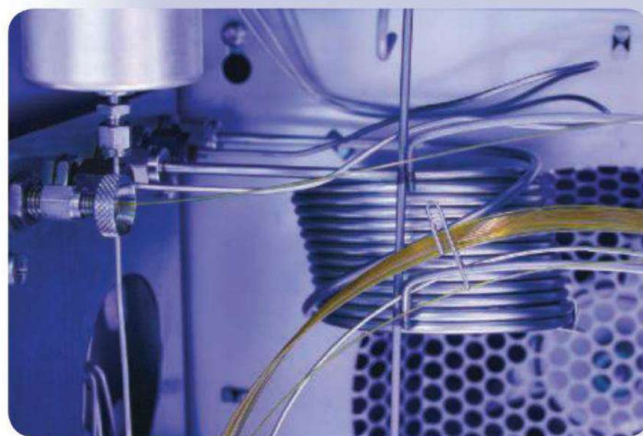
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Sample-Separation Technologies:

Improving Speed and Resolution

Many scientific tasks—including studying the composition of blood and sequencing DNA—depend on being able to separate the parts from the whole. Only then can a scientist begin to understand how the pieces build up a process. To separate a sample into its components, scientists have been continually improving traditional approaches as well as developing new ones. In some cases, the improvements speed up separations; in others, advanced techniques provide new forms of separation that improve the efficiency of the processes. Though technologies are growing increasingly sophisticated, they are also becoming easier to use.

See the full story on page 196.



Upcoming Features

Genomics: Exome Sequencing—October 11

Cell Culture: Scaling Up—December 6



Marcia McNutt is Editor-in-Chief of *Science*.

Risk

HOW MUCH RISK CAN AND SHOULD A JOURNAL TOLERATE IN PUBLISHING PAPERS THAT DESCRIBE novel findings—that is, papers that could have a profoundly positive impact within and outside the scientific community if right, but could be broadly harmful by leading investigators in wrong directions if incorrect? I recently engaged a group of the *Science* editors in a lively discussion on this topic.

We agreed that publishing papers with some such risk is a good thing. Of course, a journal would love for every paper it publishes to turn out to be perfectly correct—but not at the expense of publishing papers that are all perfectly “safe.” *Science* moves forward by communicating findings that challenge old ideas and force us to test new theories against the evidence. The key is to contain that risk.

The sources of risk and how they are best managed vary within scientific disciplines, notably in my experience between the observational fields versus the experimental fields. Experiments take advantage of controls, use multiple replicates, vary initial conditions and independent variables, and hold constant the factors that might otherwise confound results. Barring outright fabrication, results from the experiments should be reproducible to within known uncertainties. Because the experiments have been designed to test the authors’ hypotheses, there is generally a relevant result. Many of these investigations are conducted with great effort and creativity. The paper may still be risky, but the risk is generally quantifiable.

On the other hand, scientific advances that depend on new observations are inherently limited by their availability and quality. We have only one Earth to study, not multiple independent realizations and no “control” planet from which to gain statistical reliability. The initial conditions are lost in time and cannot be determined with any certainty. Astrophysicists and ecologists, for example, face challenges in which the relevant space scales are larger than any laboratory and the time scales exceed human lifetimes. The observations available are not the ideal data set for testing the most pressing questions that need to be answered. The scientists must test hypotheses with the data that they have discovered, not the information that they want to have. And yet the questions that need to be answered are too important to be ignored merely because the definitive experiment cannot be designed.

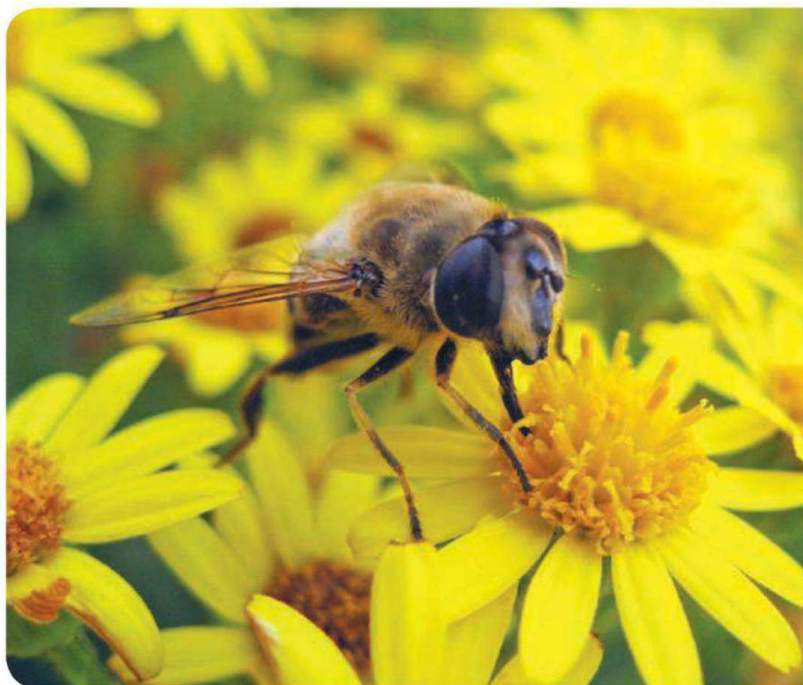
In fact, the publication of provocative interpretations and the desire to test their validity can prompt the development of new observational tools, which in turn can drive progress in observational fields. The encouragement of risk does not mean that *Science* wants submissions that are unsubstantiated by data, and clearly we must continue to strive for the highest standards in scientific peer review. But as a brilliant marine geologist once told me, “I refuse to be held responsible for prior interpretations which I have now revised based on newer and more complete information.”

I worry that the judgment of whether or not a paper carries an acceptable amount of risk can be clouded by preconceived bias—on the part of the authors, the reviewers, or even the editors. Biases make it hard to determine whether the risk that the data do not support the conclusions in the paper is real or imagined. Years ago when a decades-long debate raged in the geoscience community about whether the seismic discontinuity at 670 kilometers formed a barrier to convection in Earth’s mantle, a former mentor asserted that he was one of the few participants in the conversation who was intellectually honest because he had changed his mind as new observations had come to light. He postulated that some of his colleagues were practicing religion, not science. I urge authors and reviewers to work earnestly with the editors at *Science* to make good decisions in risky matters by examining the evidence with an open, unbiased mind.



— Marcia McNutt

10.1126/science.1242553



ECOLOGY

Conservation Pay-Off

The steady decline of biodiversity and the increasing homogenization of biotas through human influence has become a familiar theme of the 21st century. Carvalho *et al.*, however, suggest that these processes may be slowing down, at least for some groups of organisms in Europe. Focusing on assemblages of flower-visiting insects and plant species recorded in four 20-year time periods from the 1930s onward in Britain, Belgium, and the Netherlands, they show that the rates of biodiversity decline and biotic homogenization were at their greatest in the mid-20th century, a period marked by the most rapid expansion and intensification of agriculture. Since 1990, the rate of change has decelerated, indicating that conservation measures, along with the declining rate of land conversion, are beginning to have a positive pay-off for at least some of the elements of European biodiversity. — AMS

Ecol. Lett. **16**, 870 (2013).

CHEMISTRY

New Life for Cyclopropanes

Although the carbon bonds in triangular cyclopropane rings are strained, these substructures appear in numerous, reasonably stable natural and synthetic compounds. Biochemically, they tend to result from enzymatic coupling of olefins with stabilized cations. Chemists instead typically treat the olefins with transiently generated, metal-bound neutral carbenes, a strategy that broadens the versatility of substitution patterns around the ring. Mechanistically, metal activation of carbenes is loosely analogous to the pathway whereby cytochrome P450 enzymes activate oxygen, and this insight led recently to the preparation of engineered P450 variants active for synthetic cyclopropanation in aqueous solution (see Coelho *et al.*, Reports, 18 January 2013, p. 307). Coelho *et al.* have now further engineered this class of enzymes to enable carbene-derived cyclopropanation *in vivo* in *Escherichia coli* cells, despite the complete absence of a native reaction in this vein. The key mutation was replacement of a cysteine residue with serine, leading to O (rather than S) coordination of the iron active site (confirmed crystallographically). This substitution facilitated catalyst activation using endogenous NADH as a reductant. Overall activity for styrene cyclopropanation, with ethyldiazoacetate as the carbene

precursor, was even higher *in vivo* than *in vitro*, with no loss of enantio- or diastereoselectivity (favoring the *cis* product). — JSY

Nat. Chem. Biol. **9**, 10.1038/NCHEMBIO.1278 (2013).

OCEAN SCIENCE

Corals Under Threat

Rising concentrations of atmospheric CO₂ are causing the pH of surface seawater to decrease, posing a threat to coral reefs. Crook *et al.* examined how calcification of *Porites astreoides*, an important reef-building coral found in the Caribbean, is affected by the acidity of the seawater in which it grows by measuring samples

along a transect spanning a natural gradient of pH and aragonite saturation. Coral calcification rates decreased significantly as pH and aragonite saturation decreased, in a manner consistent with that exhibited by the same species in laboratory carbonate manipu-

lation experiments. This indicates that the corals were not able to respond quickly enough to prevent the impacts of local ocean acidification on their skeletal growth and development, a discouraging message considering the hopes that

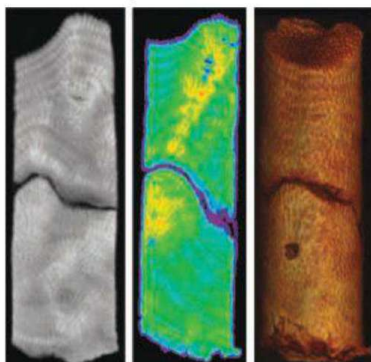
some have put on the ability of corals to adapt to future ocean conditions. — HJS

Proc. Natl. Acad. Sci. U.S.A. **110**, 10.1073/pnas.1301589110 (2013).

STRUCTURAL BIOLOGY

Form and Function

Formins are involved in regulating actin polymerization by nucleating new actin filaments and promoting elongation at the filament barbed end. In formins, a donut-shaped dimer of FH2 domains encircles the barbed end of the filament, and the FH1 domain binds profilin-actin complexes and rapidly transfers actin monomers to the barbed end. The FH2 dimer gates polymerization by transitioning between an open and a closed state, with the open state favoring actin monomer binding. Many formin-mediated actin structures experience tension, but how this affects formin function is unclear. Courtemanche *et al.* explored the effect of tension on actin polymerization induced by yeast formin Bni1p. Formin was anchored to a lipid bilayer through its N terminus, and buffer flow was used to align initiated filaments into "actin curtains." It has previously been proposed that tension might favor polymerization, by increasing the FH2 domain stepping rate, but limit the enhancement in rate provided by profilin by slowing the transfer of profilin-actin to the barbed end. Courtemanche *et al.* found the opposite—small forces slowed formin-mediated polymerization in the absence of profilin but increased the rate of polymerization in its presence. Simulations were consistent



with the proposal that tension favors the closed state of the FH2 dimer, but profilin-actin bound to the FH1 domain allosterically shifts the FH2 equilibrium toward the open state. — VV
Proc. Natl. Acad. Sci. U.S.A. **110**, 9752 (2013).

PHYSICS

Sensing Spin Order

Noncontact atomic force microscopy (ncAFM) can be used to image the spin structure of surfaces when the scanning probe (tip) is fabricated from a ferromagnetic material, such as iron. One target for imaging is the superexchange interaction between oxygen atoms on the surface of the antiferromagnetic nickel oxide, a Mott insulator. For the (001) surface of this oxide, the exchange interaction creates a (2 x 1) spin pattern. This spin structure could be imaged at cryogenic temperatures via magnetic exchange force microscopy with iron tips when a 5-tesla magnetic field was applied to stabilize the tip's magnetization. Pielmeier and Giessibl have imaged the spin structure of this surface with ncAFM without applying magnetic fields at 4.4 kelvin. They used an iron tip in a frequency modulation mode; the tip oscillated at constant amplitudes that were below 0.5 angstrom. They found that with samarium-cobalt alloy tips, the spin of the imaging atom of the tip was much more stable, and the spin signal was enhanced by a factor of 3 to 10. With this tip, a height contrast of 0.05 angstrom was seen for different oxygen atoms in the lattice that was the result of indirect superexchange interactions between the tip and subsurface nickel atoms. — PDS

Phys. Rev. Lett. **110**, 266101 (2013).

PSYCHOLOGY

Unconsciously Motivated

Recent energetic discussions in social psychology have focused on methodological aspects of research into unconscious influences on overt behaviors—for example, when participants walk like the elderly after having read passages containing words associated with old age. One consequence of these discussions would be new research programs that advance our understanding of how behavior interacts with cognitive and neural events when participants are unaware. Hepler and Albarracín have entered this arena with a study of how stimuli below the limit of perception can inhibit a behavior (not pressing a button). Without an externally observable outcome, they relied on the amplitude of an event-related brain potential

known as the P3 component, which has been shown to index inhibitory control. As their triggers for unconscious processing, they exposed participants to subliminally presented inaction (calm) and action (move) words and ascertained that participants were unaware of these primers. They found that the former set of words increased inhibitory neural activity whereas the latter set decreased it, relative to a control set of neutral words. — GJC

Cognition **128**, 271 (2013).

ECOLOGY

Just Like Home

Dispersing juveniles face the daunting task of settling in habitat that will allow them to both survive and reproduce. Our understanding of this habitat selection process is largely based on ideal free distribution (IDF) theory, which proposes that individuals assort within habitats based on the available resources, thus high-quality habitats will attract and support more animals. As with most theories, reality often contradicts IDF patterns, suggesting that other processes are at work. In particular, often individuals colonize a poor habitat even when a good habitat is



available. Piper *et al.* followed individual long-lived common loons from fledging to adulthood over 20 years. They found that dispersers preferentially colonized lakes similar to their natal lakes, particularly in terms of size and alkalinity, even when habitats of higher quality were readily available. This preference was pronounced for the first habitat selected postdispersal and waned later, when animals seemed to select for habitats that facilitated the greatest reproductive success. This temporal change suggests that familiar conditions may be beneficial to young animals as they learn survival skills and that habitat of the highest quality is only important when reproducing becomes a greater challenge than surviving. — SNV

Proc. R. Soc. London Ser. B **280**, 10.1098/rspb.2013.0979 (2013).

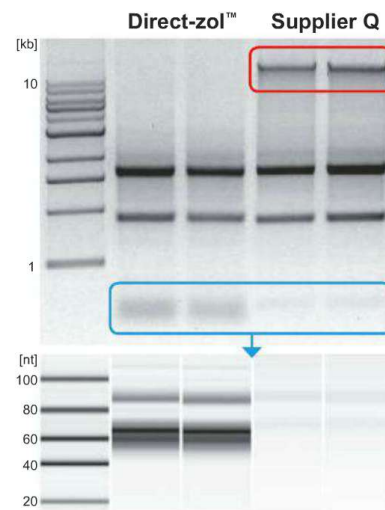
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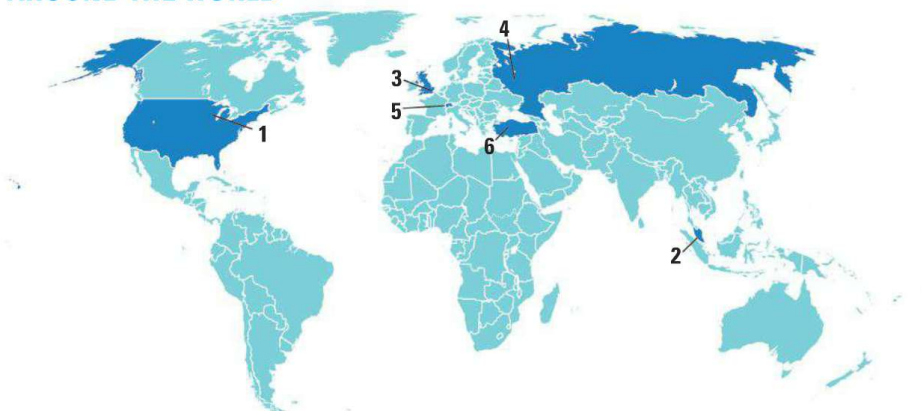
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AROUND THE WORLD



Madison 1

U.S. Stem Cell Patent Challenged

Spurred by a recent U.S. Supreme Court decision that ruled out patents on human genes (*Science*, 21 June, p. 1387), two nonprofit groups are now asking a federal appeals court to throw out the key patent on human embryonic stem cells.

The Public Patent Foundation of New York City—a leader in the gene patents case—joined with Consumer Watchdog of Washington, D.C., and Santa Monica, California, to argue that human stem cells cannot be patented because they are a product of nature. The suit focuses on research published in 1998 by biologist James Thomson of the University of Wisconsin,



Thomson

Madison. In 2006, a U.S. patent recognized Thomson as the inventor and gave the intellectual property to the Wisconsin Alumni Research Foundation (WARF). The challengers argue that WARF's patent should be rejected for two reasons: It describes a product of nature, and the discovery was "obvious" in that similar work had been done before with nonhuman stem cells.

WARF's Managing Director Carl Gulbrandsen said that the agency has "not yet" responded with a legal brief.

Kuala Lumpur 2

Evidence Mounts for Two More HIV Cures

Two HIV-infected men who had stem cell transplants to treat blood cancers have gone off antiretrovirals (ARVs), and early indications suggest that they may have cleared the

virus. This resembles the case of Timothy Brown, the first person cured of an HIV infection, who has been virus-free for more than 6 years.

Researchers from the Brigham and Women's Hospital in Boston last year reported no sign of HIV in the men following the transplants (*Science*, 3 August 2012, p. 509). But the patients were taking ARVs, which can reduce HIV to undetectable levels.

On 3 July, the team led by infectious disease clinician Timothy Henrich and virologist Daniel Kuritzkes described at a conference here how the patients stopped treatment 8 and 15 weeks ago, and neither had detectable virus in their blood. Typically, HIV levels climb high within 8 weeks after treatment stops. But, Kuritzkes cautions, "I think we need a minimum of 1 to 2 years before we can say these patients have achieved permanent remission." The researchers, who hope to do more intensive tests for the virus, stress that the costly, risky transplants have little wide-scale applicability.

London 3

Britain Boosts Health Data Research

A consortium of British funders has pledged £39 million (\$59 million) to establish the Farr Institute for health informatics research. Named for William Farr, a 19th century founder of medical statistics, the virtual institute will link main research centers in London, Dundee, Swansea, and Manchester as well as participants at 19 universities across the United Kingdom. A group of 10 funders committed £19 million to the center earlier this year, and on 3 July the Medical Research Council announced a £20 million contribution.

The effort will allow researchers to connect anonymized electronic health records from Britain's National Health Service and other data sources to analyze hard-to-study epidemiological questions such as post-approval drug side effects and the public health effects of policies such as smoking bans, says Andrew Morris, dean of the School of Medicine at the University of Dundee. "Arguably, health care is the last major industry to be transformed by the information age," says Morris, one of the coordinators of the institute.

Farr set an example in the late 1800s, however, when he developed a system to routinely record the cause of death—an innovation that led to epidemiological insights into patterns of disease and mortality.

Moscow 4

Russian Academy Reorganization Moves Ahead

The Russian Academy of Sciences (RAS) last week gained a 3-year reprieve from a government plan to merge it with two smaller academies. But Duma, Russia's parliament, endorsed several other

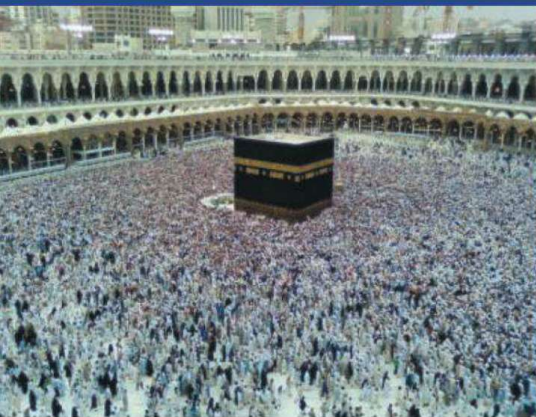


In talks. RAS head Fortov and Russian President Putin on 3 July.

changes, including stripping the academy of control over its property and real estate holdings, which many scientists believe will ultimately destroy the 289-year-old research organization.

A group of RAS academicians are hoping that academy leaders will draw up a viable alternative to the government's plans before the Duma takes a final vote on the legislation this fall. Newly elected RAS President Vladimir Fortov was picked to lead a new agency with authority to manage RAS property, but that job falls far short of Fortov's wish to be given the chance to reform RAS internally and without direct government intervention. "Previously, how the system worked was very bad, but understandable," says one scientist. "Now it is completely incomprehensible." <http://scim.ag/RASplans>

CREDITS (LEFT TO RIGHT): JEFF MILLER/UNIVERSITY OF WISCONSIN, MADISON; RIA-NOVOSTI; MIKHAIL KLIMENTYEV, PRESIDENTIAL PRESS SERVICE/AP PHOTO



Packed tight. Public health experts worry that the hajj could increase incidence of MERS.

Geneva, Switzerland 5

WHO Names MERS Emergency Committee

The World Health Organization (WHO) has named a panel of 15 experts to advise Director-General Margaret Chan on whether the Middle East respiratory syndrome (MERS) virus constitutes a “public health emergency of international concern.” The committee was expected to meet for the first time by telephone on 9 July. It is just the second such emergency panel ever created by WHO; it convened the first in 2009 to combat the H1N1 flu pandemic.

Members of the international group include Saudi Deputy Health Minister Ziad Memish; Martin Cetron, director for the Division of Global Migration and Quarantine at the U.S. Centers for Disease Control and Prevention; and Theresa Tam, an expert at the Public Health Agency of Canada.

Under International Health Regulations, declaring a global crisis would give WHO power to recommend actions, such as travel restrictions, to control the spread of the novel coronavirus, which has infected 80 people so far and killed 44. Keiji Fukuda, assistant director-general for health security and environment at WHO, says that although there is “no acute emergency,” WHO wants to be “as ready as possible.” <http://scim.ag/MERScomm>

Ankara 6

Turkish Scientists See Growing Antievolution Bias in Government

Two weeks ago, the Scientific and Technological Research Council of Turkey (TÜBİTAK), the country’s main research-funding agency, rejected a funding application for a summer workshop on quantitative evolutionary biology because “evolution is a controversial subject.” Now, the organizers are calling this the first open admis-

sion of a bias against evolutionary biology by Turkey’s conservative government. The government began blocking educational evolution websites in 2011, and recently TÜBİTAK stopped publishing books on evolution, a decision it claimed was based on copyright issues.

The workshop was to expose Turkish biology students to population genetics, game theory, and evolutionary modeling, says Erol Akçay, a Turkish evolutionary biologist at Princeton University who is one of the organizers. Akçay and his colleagues asked TÜBİTAK for 35,000 Turkish lira (about \$18,000) to cover students’ accommodation and speaker travel.



Evolving bias? The workshop’s organizers are part of Turkey’s Evolution and Ecology Network (in 2009).

Murat Özoğlu, the deputy chair of Science Fellowships and Grant Programmes at TÜBİTAK, says that the rejection was the result of objective peer review, adding that “TÜBİTAK has no reservations in supporting projects on the subject matter as it was erroneously claimed.”

The meeting will go ahead despite TÜBİTAK’s decision, Akçay says, with private donors closing the funding gap. <http://scim.ag/Turkevol>

NEWSMAKERS

Three Q’s

Japanese Prime Minister Shinzo Abe has made economic revitalization a priority, and fostering innovation is a key part of his plan. He has tasked his advisory Council for Science and Technology Policy (CSTP) with making sure that the nation’s R&D budget supports that goal. At a press briefing last week, **Yuko Harayama**,



Harayama



Seasons of Love?

As early as the 1930s, researchers noticed that children born in winter were prone to health problems: slower growth, mental illness, even early death. That observation is grounded in truth, scientists reported online this week in the *Proceedings of the National Academy of Sciences*: When it comes to low birth weight and prematurity, both linked to diverse health problems, May is the most unfavorable time to get pregnant.

Using data from vital statistics offices in New Jersey, New York, and Pennsylvania about births between 1994 and 2006, economists Janet Currie and Hannes Schwandt of Princeton University found that babies conceived in May were 13% more likely to be born premature, and gestation time was almost a week below average. For conceptions between January and May, gestation length declined by about a week before shooting back up to average length in June. This closely aligns with the time when the most patients visited the doctor for flulike symptoms, Currie and Schwandt found—suggesting that flu could cause mothers to deliver early. <http://scim.ag/pregmonth>

an executive member of the council, outlined how next year’s S&T budget, due to be unveiled at the end of August, will speed the progress of discoveries to the marketplace. Three key comments from her talk:

Why a stronger policy council is needed:

Y.H.: We need the ministries to collaborate. It is rational to have a central entity taking an overview of all of the budget related to [for example] life science and health to accelerate [turning] discoveries into products.



>>NEWSMAKERS

How management of research programs might change:

Y.H.: We are [examining] the function played by a program manager. Traditionally, once [an administrator] fixed a budget the work was finished. We need someone ... to make sure something is really moving ahead.

On getting more women in science and technology:

Y.H.: I am [the first woman] in a permanent position at the CSTP. The most important thing [about having] women ... in the decision-making sphere is that they may bring in new thinking. What I will try to do is promote women ... putting pressure on the universities, but the same should be done in industry.

FINDINGS

Weak Immune System Toughens Malaria Parasite

The arms race between pathogens and their host can heat up even if the host is a wimp. An experimental evolution study in mice



Virulence boost. Researchers saw parasite evolution in immunocompromised mice.

has found that malaria parasites infecting rodents with weakened immune systems evolve increased virulence.

Andrew Read and Victoria Barclay of Pennsylvania State University, University Park, tested this by first giving a pair of mice antibodies that disabled a key immune molecule, the CD4 receptor, and then infecting them with a mouse malaria parasite. Then, each week for 21 weeks,

they infected a new pair of immunocompromised mice with parasites taken from the immunocompromised mice infected the week before. Finally, to compare the virulence of each week's parasites, they infected healthy mice with the pathogens; parasites from week 21 grew faster and caused more anemia and weight loss than parasites from week 10, Read reported last month at Evolution 2013 in Snowbird, Utah.

Read is concerned that the rise in number of HIV-infected and other immunocompromised people might hasten the evolution of pathogens into deadlier forms. Although Read's mouse experiment "is a very elegant and sobering study," says Daniel Bolnick, an evolutionary biologist at the University of Texas, Austin, "we certainly can't take it for granted that biological patterns in mice can be extrapolated to humans."

Random Sample

Name Those Moons

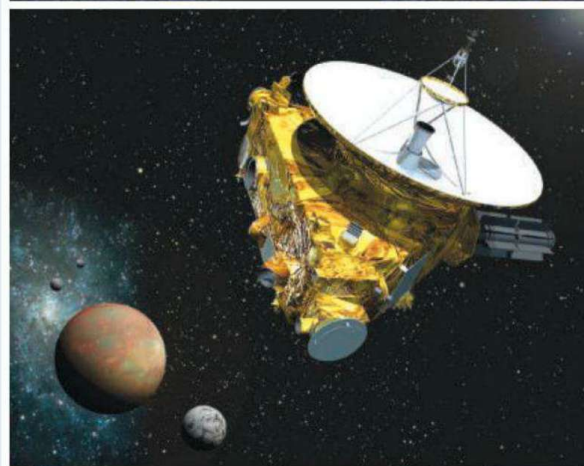
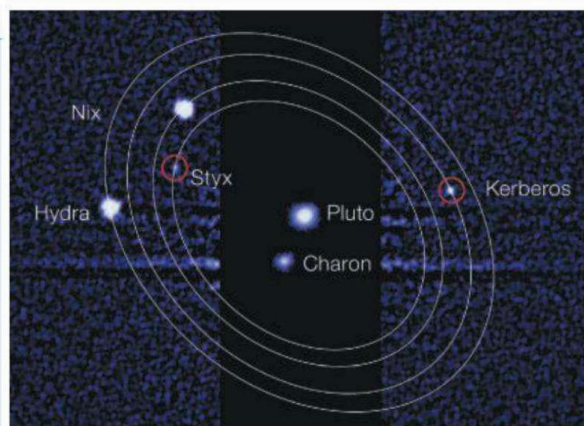
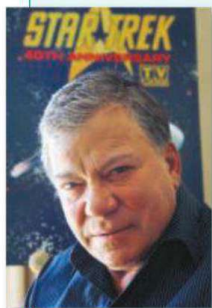
Pluto fans should hail planetary scientist Mark Showalter of the SETI Institute in Mountain View, California. Not only did he sign a petition opposing Pluto's demotion from planet to dwarf planet, but he also discovered two new moons circling the far-off world, thereby boosting its planetary cred.

But what to name the new satellites? "I got hundreds of e-mails from people I don't know," Showalter says. So planetary scientist Alan Stern of the Southwest Research Institute in Boulder, Colorado, suggested an Internet poll. Names had to refer to the underworld, as Pluto was its mythological god.

With 450,324 votes cast, the winner was one that actor William Shatner championed: Vulcan, home of *Star Trek's* Mr. Spock. Runners-up included Cerberus, Styx, and Persephone.

Showalter's team chose the top two, but officials vetoed Vulcan, the Roman god of fire and forges. "Pluto and Vulcan didn't cross paths much," Showalter admits. Cerberus, Pluto's three-headed dog, is already the name of an asteroid but received approval in its Greek form, Kerberos. Styx also made the grade as the goddess of the river dividing the world of the living from the underworld. Showalter says: "I am very happy with the outcome."

Kerberos and Styx join Pluto's three other moons, Charon, Nix, and Hydra, the last named for a monster whose nine heads signify that many consider Pluto to be the ninth planet. Indeed, the emblem for NASA's Pluto-bound New Horizons spacecraft (bottom right) is nine-sided, and Showalter says that he'd be "almost shocked" if it doesn't find yet more moons when it reaches its target in 2015.





SEISMOLOGY

Some Earthquakes Warn That They Are About to Strike

The bad news that injecting wastewater deep into the crust can set off earthquakes has now been leavened by a bit of good news. In the past few years, the frequency of moderate-sized earthquakes has surged in parts of the United States where wastewater from “fracking” for gas and oil is pumped into the deep earth for disposal. Now, seismologists have found that some of the largest quakes induced by deep injection are preceded by a warning sign: a distinctive swarm of smaller tremors.

The practical value of the discovery is limited. It applies to earthquakes linked to fluid injection, not as yet to large natural quakes along faults such as the San Andreas. Not all injection-related quakes ever telegraph their moves. And the warning depends on the chance occurrence of large, distant quakes that tickle local faults into low-level activity shortly before injection induces a larger quake. But to researchers who have searched in vain for any kind of earthquake warning sign, the finding is a milestone.

“We’ve been looking for this for years,” says seismologist Emily Brodsky of the University of California, Santa Cruz. “This is one of the holy grails—a way to probe the state of stress of the crust. This shows you can do it.”

Seismologists have long recognized that deep injection can induce earthquakes. The injection increases the fluid pressure along a fault that is already under stress, which can counteract the forces squeezing the fault together and make it more likely to rupture.

Sure enough, in the past few years, quakes of magnitude 4 and 5—alarming but barely destructive—began to shake sites of deep injection in the eastern two-thirds of the United States (*Science*, 23 March 2012, p. 1436). With public concern soaring, seismologist Nicholas van der Elst of Columbia University’s Lamont-Doherty Earth Observatory in Palisades, New York, and colleagues decided to search the seismic record near places where deep injection has been physically linked to sizable quakes for any premonitory signs.

Aiding their search were the 400 traveling seismic stations of the USArray (*Science*, 14 June, p. 1283), which boosted the available data by supplementing the fixed seismic stations across the United States. Van der Elst and colleagues analyzed the data using a technique that matches the seismograph squiggles of quakes that strike the same spot, so they could detect patterns in earthquake times and locations.

As they report this week in *Science* (p. 164), the warning sign of an impending human-induced earth-

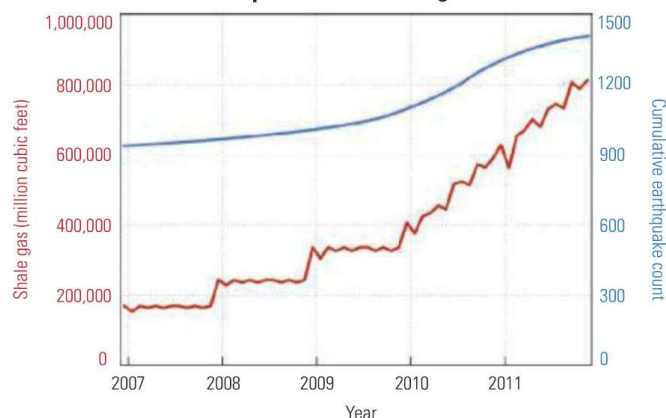
Shaken by injection. Deep fluid injection induced an alarming quake beneath Youngstown, Ohio.

quake turned out to be swarms of smaller quakes set off by passing seismic waves from large, distant quakes. During the 10 days after the March 2011 great Tohoku-oki earthquake off Japan, almost a score of quakes ranging up to magnitude 3.8 struck near Snyder, Texas, where an injection-induced magnitude 4.5 later struck on 11 September of that year. A great quake before Tohoku-oki and one after the induced Snyder quake triggered no swarms near Snyder. A similar seismic pattern related to distant triggering quakes appeared around three large (magnitude 5.0 and larger) human-induced quakes near Prague, Oklahoma, in November 2011. The pattern, weaker but still identifiable, also was found around the human-induced seismic swarm at Trinidad, Colorado, in August 2011 that ranged up to magnitude 5.3.

Van der Elst and colleagues analyzed records from the areas of three other injection-induced quake swarms, but they found no precursory triggering by large distant earthquakes. Because all three swarms struck not long after injection began, the researchers suspect there simply happened to be no distant quakes large enough to trigger a warning swarm during the short time when the sites were vulnerable.

“It might not happen every time,” says van der Elst of the remotely triggered warning quakes, but “where it does happen indicates you should be careful.” Seismologist William Ellsworth of the U.S. Geological Survey (USGS) in Menlo Park, California, agrees. It would be “wise to be alert to the

Earthquakes and Fracking Wastes



Quakes followed fracking. Natural gas production (red) from fracking shale soared as central U.S. seismic activity accelerated (blue, smoothed approximation).

possibility of [remote] triggering,” he says. “That’s a sign that it might be prudent to back off on injection pressures and rates.”

Researchers say that it makes sense that a passing seismic wave would elicit quakes from a fault already weakened by fluid injection. In waterlogged crust riddled with faults that are on the verge of failing—such as southern California’s Salton Sea geothermal region—passing seismic waves ever so slightly pump up fluid pressure and trigger small quakes just as deep injection is thought to do.

Remote triggering won’t likely be incorporated into deep injection regulations anytime soon—the science is hardly mature—but drillers doing deep injection will no doubt be taking note. They already know a surge in seismicity at the start of injection is a bad sign. And the hubbub over human-induced quakes has accelerated efforts to treat fracking wastes for surface disposal or to reuse them to cut down on the volume requiring deep disposal.

Remote triggering could also be important for anticipating quakes that pop off on

their own without human help. But it won’t be *the* holy grail of earthquake prediction, researchers say. Remote triggering, notes seismologist David Hill of USGS in Menlo Park, requires not only a well-timed distant quake of magnitude 7 or above, but also free-flowing fluids in and around the fault of interest. The San Andreas fault, for one, seems to lack free-flowing fluids, perhaps because of the unfavorable orientation of cracks around that type of fault. In any case, no remote triggering has ever been seen on the San Andreas.

—RICHARD A. KERR

EVOLUTION

Field Test Shows Selection Works in Mysterious Ways

SNOWBIRD, UTAH—From a distance, Rowan Barrett, a slight man with a thick black beard, doesn’t really stand out in the crowd. But his peers think that he does. For the past 2 years, Barrett has swept the main awards for young evolutionary biologists, capping off his run last month at the Evolution 2013 meeting with the Dobzhansky Prize from the Society for the Study of Evolution. His claim to fame: daring to do experimental evolution on a grand scale to examine how selection shapes an organism’s whole genome.

“He’s proven that he’s not afraid to dive in and test hypotheses in a natural setting in situations where it was previously thought that it would be too difficult to test,” says Robin Hopkins, an evolutionary biologist at the University of Texas, Austin. “He has incredible persistence.”

Barrett works in the Sand Hills of Nebraska, where mice living on the light-colored dunes are lighter in color than those in the surrounding prairie. Researchers have long assumed that the dune mice lightened up to become less visible to airborne predators such as owls, and studies have pinned down changes in a pigment-related gene called *Agouti* in these mice (*Science* 28 August 2009, p. 1095; 15 March, p. 1312).

That tidy tale left Barrett dissatisfied. “This has been one of the longest-standing adaptive stories in evolutionary biology, but the hypothesis that cryptic coloration is adaptive has never been directly tested in deer mice under natural conditions,” he says.

It has now. Barrett told the audience at the evolution meeting that his preliminary results suggest color does protect the dune mice—but that other genes also help the mice thrive. The results show that “there are other things that played a role in adaptation,” says David



Tracking evolution. Using big field enclosures, researchers are monitoring the evolution of deer mice.

Reznick, an evolutionary biologist from the University of California, Riverside.

Barrett began the work in 2010, as a postdoc with Harvard University’s Hopi Hoekstra. He and his colleagues trapped hundreds of Nebraska deer mice living on light and dark ground, characterizing their coats, sampling their DNA, and marking them for follow-up studies. They then released sets of about 100 mice—half from each environment—into six 50-meter by 50-meter enclosures in the Sand Hills and on dark soil nearby. Steel fences 1.5 meters tall, extending 60 centimeters underground to prevent burrowing, kept the mice from escaping, but did not protect them from airborne predators. Every few months for 2 years, the researchers trapped all the mice in each enclosure, tracking which were still alive and monitoring shifts in the frequency of different versions of the rodent’s genes.

Not surprisingly, dark mice did better on the dark soil, and light mice did better on the light sand. On a genetic level, Barrett’s anal-

ysis confirmed the importance of the *Agouti* gene—multiple differences in its sequence were either favored or selected against in the various enclosures. “Selection is on a fairly broad region of the *Agouti* gene,” says Dolph Schluter, an evolutionary biologist at the University of British Columbia, Vancouver. “It’s not just the case of one mutation being favored.”

But many genes not related to pigment also changed over the course of the experiment. The analysis revealed “a potentially large number of genetic regions responding to selection,” Barrett reported. He doesn’t know what traits those changes affect, but he’s investigating each one—and suspects some have nothing to do with surviving predation. They might make a mouse healthier overall, or better able to find food, for example.

“A lot of what we know about selection is very crude,” Schluter says. “When we start to do experiments we are surprised to find stuff happening that we didn’t anticipate.”

—ELIZABETH PENNISI

CREDIT: ROWAN BARRETT

U.S. NATIONAL LABORATORIES

As Budgets Tighten, Washington Talks of Shaking Up DOE Labs

It's back to the future for Washington policymakers concerned about the U.S. Department of Energy's (DOE's) sprawling network of 17 national laboratories.

For the third time in 2 decades, members of Congress and DOE leaders are considering a major inquiry into whether the government is getting its money's worth from an annual investment of \$12 billion. The goal: to make sure the labs are conducting high-priority research and not wasting resources on duplicative programs. But don't hold your breath for dramatic change. Few government programs are more entrenched than DOE's labs, and previous reform efforts have produced relatively incremental shifts.

Despite that history, concerns about tight budgets and the need for greater efficiency have prompted policymakers to take up the challenge. This week, the science committee for the U.S. House of Representatives held a hearing on a new report from an unusual trio of think tanks that calls for a radical makeover of lab practices and DOE's senior leadership. On 27 June, a Senate spending panel voted to establish a nine-member national commission that would recommend ways to improve lab operations—including the possibility of closing or consolidating facilities. And Energy Secretary Ernest Moniz, a Massachusetts Institute of Technology physicist and former DOE honcho who took up his new post in May, has said that he is considering his own internal review of the labs as part of a broader possible shakeup of top DOE management.

Outside groups are providing plenty of debating points. The U.S. National Academies and Congress's watchdog arm, the Gov-

ernment Accountability Office, are separately finalizing an array of studies that includes analyses of controversial cost overruns in lab projects and management problems at the three weapons labs.

"These are issues that just don't go away," says David Garman, a former DOE undersecretary who was involved in a major effort to assess lab performance under President George W. Bush in the mid-2000s. But, he says, "they appear to be ripe for a fresh look."

Although the first lab was created in 1931 to do physics research, most were founded in the 1940s and 1950s to help build nuclear weapons (see chart). Today, the system includes outposts as small as Iowa's \$30-million-per-year Ames Laboratory, which focuses on energy and materials research, and as large as New Mexico's Sandia National Laboratory, which spends some \$4 billion annually on weapons and security studies. Almost all are managed for the government by private companies or coalitions of universities and non-profit groups.

As the network has grown, however, so have complaints about bureaucratic red tape, duplication of effort, and spectacular cost overruns on high-profile projects. Those issues came to a head in 1995, when Motorola executive Robert Galvin was asked to lead a commission that examined ways to downsize labs considered bloated by Cold War budgets. The so-called Galvin Report stopped short of recommending lab closures, but did lead to other changes. In 2003 and 2005, the George W. Bush administration and Congress followed up with their own committees and reforms, including the creation of a new DOE undersecretary for science to separate oversight of the 10 labs focused on basic research from those handling weapons, waste management, and applied energy research.

Although the changes led to some streamlining, they didn't satisfy many critics, and now, amid tight budgets, Moniz and Congress appear ready to take another look. "The concern becomes: 'What are you going to have to cut if you want to do something new?'" says physicist Michael Lubell, head of public affairs for the American Physical Society.

Another perennial issue is whether DOE has wrapped the labs in too much red tape. "Decisions that should be made by lab scientists can take weeks to go through layers of middle management," says Matthew Stepp of the Washington, D.C.-based Information Technology and Innovation Foundation. Last month, his centrist group forged an unusual alliance with analysts from the conservative Heritage Foundation and the liberal Center for American Progress to issue a report that calls for changes designed to reduce "micro-management" and ease lab collaborations with industry. "The labs have been largely running on autopilot for too long," argues the report, which was scheduled to be a major agenda item at the 11 July House science panel hearing. "A jolt to the system is needed now more than ever."

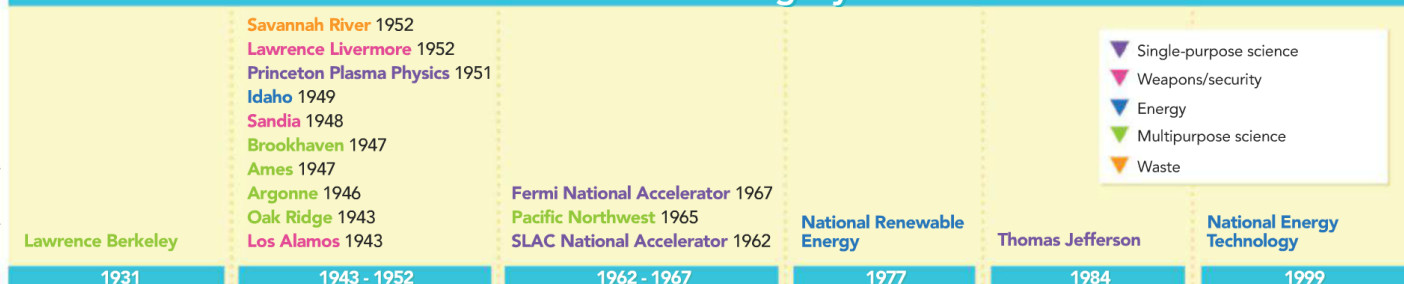
The shock therapy could start, Stepp says, by undoing the 2005 decision to create the undersecretary for science. Instead, a new position would oversee and coordinate research at both the science and energy labs. (The weapons labs have their own undersecretary.) That's something that Moniz could do now, without congressional approval, insiders note. But more dramatic changes, including consolidating or closing labs, would require buy-in from lawmakers and the labs' often vociferous constituents.

The Senate's idea for a national commission (which would need approval by the House and the White House) could be a first step in building such consensus, says Garman, now a consultant in Washington, D.C. "There's always a certain amount of turbulence surrounding the labs," he says, but the current level "does suggest some kind of review needs to happen."

—DAVID MALAKOFF

Nuclear explosion. Many of the Department of Energy's national laboratories were founded to help build nuclear weapons during and after World War II.

Cold War Legacy



EVOLUTION

Diverse Crystals Account for Beetle Sheen

SNOWBIRD, UTAH—When J. B. S. Haldane said decades ago that God must have had an inordinate fondness for beetles, the British biologist might have had the iridescent ones in mind. Not only are beetles among the most diverse group of animals on Earth, but some also rival the most flamboyant birds and dazzling coral reef fish with their metallic sheens. Researchers have now traced the evolutionary history of this iridescence in one group of beetles, weevils, and identified an unexpectedly varied assortment of complex light-altering crystals in the insects.

The new work, reported last month at Evolution 2013, also shows that these diverse crystal architectures all share a single origin in these beetles' evolutionary past. Weevils "have developed high crystal diversity from a relatively modest tool kit," says Ainsley Seago, a systematist with the Australian National Insect Collection in Canberra, who led the work.

Among organisms, colors arise several ways. Pigments are responsible for the cardinal's bright red plumage and the larkspur's deep blue flower. Some animals dress up without pigments, relying on relatively simple, layered structures that break up incoming light and reflect back a rainbow of colors that are seen only from the right angle. But in 2003, researchers discovered what they described as an "opal" inside tiny scales on the surface of a multicolored weevil, creating colors that are visible from all angles. True opals are amorphous crystals that lack a distinct lattice structure, but many weevils turn out to rely on nanostructures known as three-dimensional photonic crystals, which have a more regular geometry that influences the path of light. Made of the polysaccharide chitin, the main component of the insect exoskeleton, the crystals fill up flattened scales on the outside of a weevil's exoskeleton.

In a survey of 300 weevil species, representing the major lineages of this large group of beetles, Seago and her colleagues found iridescence in weevils of all different shapes and sizes. She then determined the geometries of the crystals responsible for the colors by bombarding them with high-energy x-ray beams and examined scales from 64 of the species with a scanning electron microscope. The result: Not only was iridescence scattered throughout the weevils, but their crystals were also arranged in diverse geometries—diamond, curved, etc. Such evidence suggested that the trait had

originated multiple times in weevil evolution.

Weevil DNA told a different tale, however. Seago and her colleagues compared the DNA in 64 of the 300 species and built a family tree that showed how the species were related and which lineages arose first. She mapped the lifestyle of each beetle species,



Dazzling. Weevils have a variety of photonic crystals in their scales for generating bright colors.

its crystal type, and the color it generated onto the tree as well and from that, pieced together a compelling evolutionary story.

Several older lineages of weevils, including the fungus and pine flower weevils, have

what appears to be the evolutionary precursor to photonic crystals: a spongy matrix that scatters light in all directions, making flat white spots, Seago reported. White speckles were likely useful for camouflage in early weevils, which lived in rotting wood, soil, and fungus. According to Seago, the first true crystals appeared in the last common ancestor of the broad-nosed weevils.

The crystals are arranged in a diamond lattice, like the carbon atoms in a diamond—a geometry that usually generates a greenish sheen. This diamond geometry emerged when weevils began to inhabit live plants. "When they began to hang out on leaves, there was pressure to be cryptic," so crystal-toting green iridescent weevils had the edge, Seago said.

The broad-nosed weevils rapidly diversified, and their crystal geometries got more elaborate, her analysis indicates. Curved, or gyroid, lattices appeared, as did cubic ones. A few species have quasi-ordered nanospheres akin to the nanostructures that make sunscreens white, or combinations of diamond and gyroid patterns, she reported.

"It's really exciting that these evolved from a single origin," says Rafael Maia, a graduate student at the University of Akron in Ohio who studies iridescence in birds. "I don't think there's anything comparable to the diversity of the evolved structures that these [beetles] use."

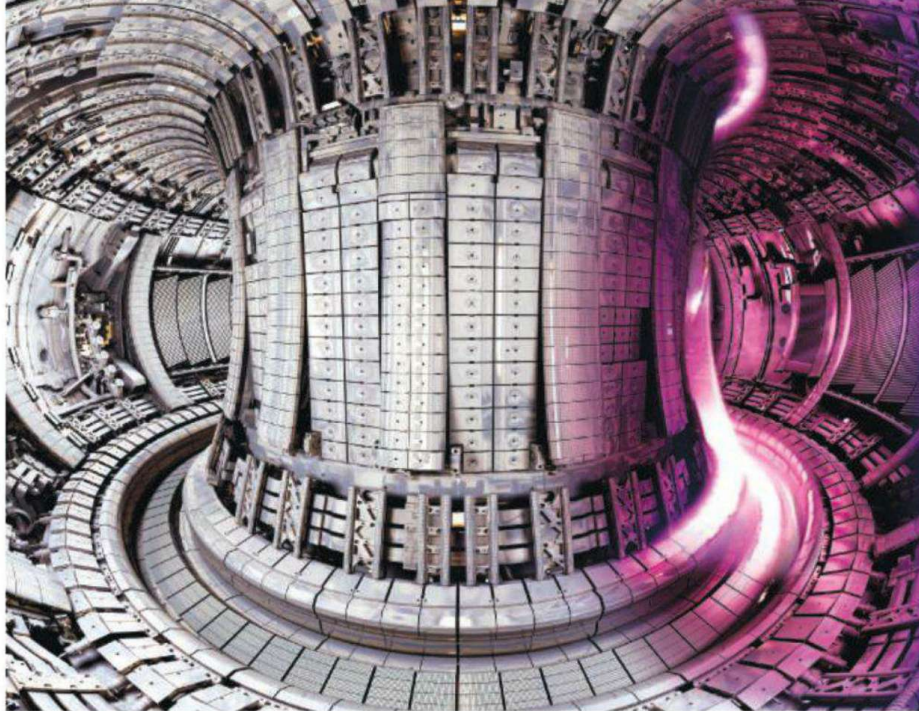
In trying to make sense of the various types of iridescent structures, Seago's University of Oxford collaborator Vinod Saranathan realized that they represent the full range of structures that form when structures like liquid crystals self-assemble in a solvent. Seago speculates that during development, internal membranes in the scale self-assemble into these different shapes, serving as temporary scaffolding for the deposition of chitin. If that's the case, "these are naturally forming through spontaneous organization," says Douglas Emlen, an evolutionary biologist at the University of Montana in Missoula. "That's a cool mixture of physics and geometry with biology."

Seago is now trying to collect immature weevils to verify how the crystals arise. "If we learn more about the formation processes of these structures, we may be able to synthesize sophisticated photonic materials from eco-friendly, biodegradable, nontoxic polysaccharides instead of the metals and plastics that are currently used," she says. If so, materials scientists could grow inordinately fond of the beetles, too.

—ELIZABETH PENNISI

CREDIT: ANSLEY SEAGO

Online
sciencemag.org
 Podcast interview
 with author
 Elizabeth Pennisi (http://scim.ag/pod_6142).



NUCLEAR FUSION

JET Fusion Reactor Passes 30 And Plunges Into Midlife Crisis

The world's largest fusion reactor, the Joint European Torus (JET), marked its 30th anniversary late last month under a cloud: It may not have many more birthdays to celebrate. As the European Union struggles to pay for its share of the burgeoning cost of JET's successor—the International Thermonuclear Experimental Reactor (ITER), now under construction in France—it is considering closing JET in 2018 unless some of the non-European partners in ITER come to the rescue. “The general consensus in the community is to allow JET to operate until at least 2018,” says JET leader Francesco Romanelli. “After that, it depends on ITER's needs and the interest of other international partners.”

JET and ITER are both experimental reactors that seek to generate power by nuclear fusion—a technically challenging process fundamentally different from the fission that drives conventional nuclear power stations. So far, no fusion reactor has produced more energy than it consumes. JET has come the closest, reaching 70% of breakeven in 1997. ITER aims to produce at least 10 times as much energy as it consumes.

Designed and built in the 1970s and early 1980s in Culham, U.K., JET is a doughnut-shaped reactor known as a tokamak, which uses powerful magnetic fields to contain its fuel: an ionized gas, or plasma, of hydrogen isotopes heated to 100 million kelvin. The fields keep the plasma from touching the walls of its container and melting the

lining. Particle beams and radio waves heat the plasma; JET also bristles with diagnostic devices to probe what is happening in the hostile environment of the tokamak's interior.

No one expected JET to last this long. “JET was designed with significant margins, particularly in size, and that's the reason why it is still open today,” András Siegler, director of energy research in the European Union's directorate-general for research and innovation, told the anniversary event. It was originally slated for closure in the 1990s, but researchers upgraded it to withstand longer fusion pulses, and in 1997 JET made a record-setting fusion pulse that generated 16.1 megawatts of power. Between 2009 and 2011, JET researchers changed the lining of the reactor vessel to the same combination of beryllium and tungsten proposed for ITER. Since then, researchers have been studying how the so-called ITER-like wall affects plasma performance—so far with generally positive results.

Researchers are eager to keep JET running because among all the world's tokamaks, it is the closest to ITER in size, shape, and plasma conditions. It is also the only tokamak now equipped to operate with the same mixture of deuterium and tritium that ITER will use—a fuel that is radioactive and requires careful handling but also yields the most energy. This month, researchers will start testing “scenarios with pulses relevant to ITER,” says Guy Matthews, JET's task force leader for the ITER-like wall. “We're going to use all

Healthy glow. A composite picture of JET's interior showing what it looks like containing a glowing plasma.

the power we can get and make it as ITER-relevant as possible.” In 2017, they plan a series of deuterium-tritium burning experiments to try to study how best to operate ITER when it comes online.

What happens after that depends partly on money. Right now, details of the E.U. budget for 2014 to 2020 are still under deliberation. But signs are that the fusion budget will not be generous—and JET must share the pot with various small fusion experiments as well as Europe's 45% share of ITER, whose €16 billion projected cost has already swelled to several times the original estimate. “The ITER budget is the elephant in the room,” says one fusion researcher who asked not to be named.

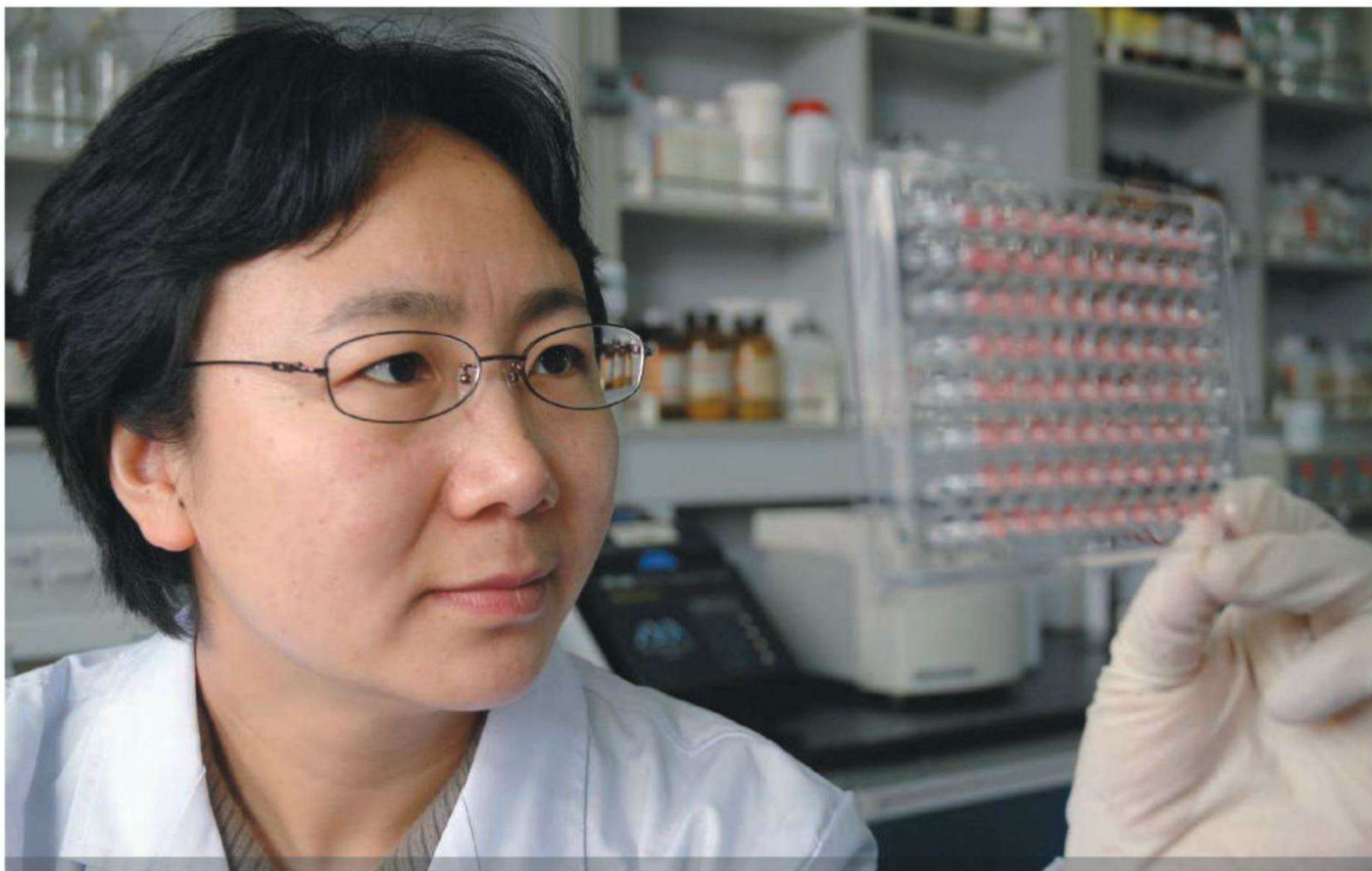
The prospect of closing JET as early as 2018, 2 years before the planned startup of ITER, worries many fusion researchers. Some argue that any delay in ITER could cause some fusion researchers to leave the field. Some also say that it would be valuable for the two reactors to overlap, so that new approaches could be tried out on a smaller scale on JET without disrupting the ITER schedule. “The thinking has been that we need a facility to train a team to operate ITER,” Matthews says.

Officials are now testing the water to see if Europe's fellow ITER partners—China, India, Japan, Russia, South Korea, and the United States—might join a new international consortium to run JET from 2018. JET's average annual running cost of only €60 million makes it a bargain, they argue. But austerity and ITER construction are also straining fusion budgets in many partner nations outside Europe.

One experiment already proposed could extend JET's life: an effort to combat an instability in fusion plasmas called edge-localized modes (ELMs), which can release potentially damaging bursts of energy. Indian researchers collaborating with JET have drawn up preliminary plans to equip the reactor with ELM-taming magnetic coils in order to test them under ITER-like conditions. If the plan is funded by some combination of ITER partners, the coils could be installed in 2016 and put through their paces between 2017 and 2020 at the same time as the D-T experiments.

“We cannot exclude other things coming up that require testing on JET,” Romanelli says. “JET is the best testbed for experiments on ITER. But when ITER starts, we should focus on ITER.”

—DANIEL CLERY



Veterinarian-in-Chief

As China's point person for avian influenza, Chen Hualan is spearheading studies on deadly strains—while fending off critics who say she's playing with fire

HARBIN, CHINA—On Saturday, 30 March, Chen Hualan was in a meeting in Beijing, pulling a characteristic weekend work session, when a tense call came through on her mobile. It was an official in China's Ministry of Agriculture, reporting that people were coming down with a novel bird flu virus. The Chinese Center for Disease Control and Prevention (Chinese CDC), Chen learned, had confirmed three cases, two in Shanghai and one in Anhui province. The cases appeared in late February and early March; Chinese CDC had subsequently isolated the virus and sequenced it. Researchers identified it as H7N9, a virus not previously detected in humans.

As the head of China's National Avian Influenza Reference Laboratory, Chen leapt to action. She'd been the go-to person for animal testing during periodic outbreaks of an earlier deadly bird flu, H5N1, and she knew that a novel virus infecting humans

was a very serious matter. After she hung up, she dispatched staff members to Shanghai and Anhui to collect samples from poultry markets and farms. On 31 March, Chinese authorities announced the virus to the world.

Within a few days, Chen's team had sequenced H7N9 strains isolated from chickens and pigeons. The results came back at 11 p.m. on Tomb-Sweeping Day, a national holiday in China that went unobserved in the Harbin lab. Chen sent the sequence data to the agriculture ministry, recommending the immediate closure of poultry markets in areas with infections. But her work had just begun. For the next month, as human cases piled up and poultry samples poured in, Chen's lab worked around the clock, testing 10,703 samples in total. Not until May did Chen get more than 5 hours of sleep a night.

The 44-year-old virologist didn't need to impress her colleagues. "Chen's massive

body of work is of outstanding quality," says Ron Fouchier, a virologist at Erasmus MC in Rotterdam, the Netherlands. But controversy soon engulfed her. After news of China's human cases went public, Chen started receiving e-mails from scientists around the world asking for samples of the virus isolated from birds. China had come under fire during past outbreaks for dragging its feet. But as head of a World Organisation for Animal Health (OIE) reference lab, she had committed to sharing the virus with other labs in a network run by OIE and the Food and Agriculture Organization (FAO), and the implication in the scientists' e-mails that she was unwilling to pony up samples irked her. "They thought we were hiding something," she says. Chen replied to the e-mails that she wasn't holding anything back: "I am also looking for the virus," she wrote. But suspicions die hard.

A quick ascent

Like many senior Chinese scientists, Chen did not choose her calling. She grew up in a small city in Gansu, a poor province in western China. When her score on China's college entrance examination did not secure one of her preferred majors—she had hoped to study medicine—she ended up studying

CREDIT: HARBIN VETERINARY RESEARCH INSTITUTE

Flu fighter. Chen studied avian influenza in China back when “nobody cared.”

veterinary science in Lanzhou, the capital of Gansu. In 1994, her Ph.D. research took her to Harbin, a major city in far northeastern China, near the Russian border, where winter temperatures can plummet to -30°C , and an army of workers uses straw brooms to sweep snow off the streets.

When she arrived at the agriculture ministry’s Harbin Veterinary Research Institute, she asked her supervisor, Yu Kangzhen, what she should research. “Whatever—as long as it’s on influenza,” he told her, she recalls. Infectious diseases were a top priority in China, yet, “nobody cared about flu,” she says. But Yu knew that avian influenza had caused outbreaks in poultry in several other countries and that Chinese farms were lax on biosecurity. If a highly pathogenic virus emerged in Chinese birds, he believed, it would spell trouble.

Yu had imported a few flu strains from an OIE reference lab in the United Kingdom, and Chen and four colleagues got to work on vaccine development. “We couldn’t do any basic research because we didn’t have too many strains at that time,” she says. Things changed in 1996, when the team isolated H5N1 from a farmed goose in Guangdong province. The agriculture ministry entrusted the lab with surveillance, which became critical the following year, when a 3-year-old boy in Hong Kong became the first person infected with H5N1. He died. That year saw 17 more cases, including five fatalities. Suddenly, bird flu went from a research backwater to a global preoccupation.

In 1999, Chen wrote Kanta Subbarao, who then headed up molecular genetics research at the U.S. CDC in Atlanta, asking to work in her lab as a postdoc. Subbarao was studying the H5N1 strains that had emerged in Hong Kong. “We were having to get a crash course in avian influenza,” Subbarao says. The Hong Kong viruses had acquired the hemagglutinin gene, key to their ability to bind to and infect cells, from the Guangdong goose strain. Chen’s familiarity with the strain snared her a job.

CDC’s working conditions wowed Chen. Back then, a scientist looking to use a PCR machine in Harbin had to sign up 2 to 3 weeks in advance. In Atlanta, every scientist had a machine. Chen worked on vaccines for H5N1 and H9N2, learning a technique for developing vaccines called plasmid-based reverse genetics. Instead of waiting to obtain plasmids developed by other labs, Chen decided to make her own—an incred-

ibly time-consuming process, says Subbarao, who is now at the U.S. National Institute of Allergy and Infectious Diseases. Chen, she says, “is not at all intimidated by the amount of work that she takes on.”

After 3 years in Atlanta, Chen, at the tender age of 33, returned to Harbin to head the national avian flu lab. She saw it as a chance to catapult into a prominent position in flu research. But her husband and son had come with her to the United States, and her decision to move the family back to China raised eyebrows. People “asked me, ‘Where are you going to go, Shanghai or Beijing?’ And I said, ‘Harbin.’” Colleagues, she says, “thought I was crazy” to return to the frigid outpost.

The lab, housed in a pre-1949 building with the feel of a sanatorium, turned out to be the center of action. In 2003 and 2004, human H5N1 cases emerged in Hong Kong, mainland China, South Korea, Thailand, and Vietnam, while poultry outbreaks spread across Asia. Prior to those outbreaks, scientists at the avian flu lab had isolated more than 20 H5N1 strains from apparently healthy ducks. But the staff had been without a director for 2 years before Chen arrived; lacking guidance, they stored the specimens in a freezer. Under Chen’s direction, they got to work sequencing strains and testing virulence in mice. They discovered that the nastiest strains were those isolated from more recent samples. In an influential paper published in the *Proceedings of the National Academy of Sciences* in 2004, the team showed that H5N1 had progressively acquired the ability to kill mammals—meaning a longer list of H5N1 genotypes than previously thought could threaten people.

Chen’s research interests and staff have since multiplied. Her 80-person team has, among other things, developed an avian vaccine against Newcastle disease—a contagious virus that can cause conjunctivitis and flulike symptoms in humans—and an avian H5N1 vaccine now used worldwide. The lab joined OIE’s reference network in 2008 and in March was designated an FAO reference center, a badge of global excellence awarded to a select number of labs. The institute that houses the lab is preparing to move to a gleaming new 260,000-square-meter campus. The differences between research conditions in China and in the West are “getting smaller and smaller,” Chen says. She may even have an advantage over many colleagues, given the abundant resources and staff members she enjoys. Pondering what her path would have been had she stayed in Atlanta, Chen says: “Maybe a postdoc for many, many years?”

H7N9: A Bird’s Eye View

This spring, researchers scrambled to contain a novel and deadly virus



Saturday, 30 March

Chen gets urgent call: People are contracting a novel bird flu virus



Sunday, 31 March

Chinese government announces three human H7N9 cases in Shanghai and Anhui



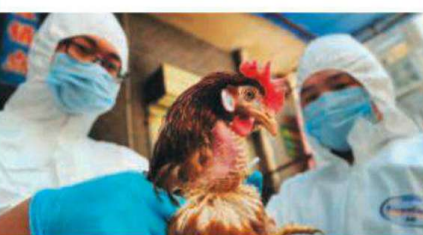
Thursday, 4 April

Chen’s team sequences first H7N9 strains isolated from chickens and pigeons



Saturday, 6 April

Shanghai government closes all live poultry markets



April

Chen’s lab works around the clock to test 10,703 poultry samples for presence of virus

Thursday, 20 June

Shanghai reopens some poultry markets

CREDITS (TOP TO BOTTOM): CDC/C. S. GOLDSMITH AND T. ROWE; WANG QI ZI/MAGINECHINA/AP PHOTO; LIU JIANHUA NJ/MAGINECHINA/AP PHOTO; ZI XIN/MAGINECHINA/AP PHOTO

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Risky business?

On 2 May, Chen and colleagues published a controversial paper online in *Science* in which they argued that the H5N1 virus could set off a pandemic (*Science*, 21 June, p. 1459). So far, human-to-human transmission has been limited. Of the 375 deaths attributed to H5N1 as of 4 June, in nearly all cases victims are known to have caught the virus from infected birds. Chen set out to answer a question that others—most notably Fouchier, and Yoshihiro Kawaoka of University of Wisconsin, Madison—had probed as well: What changes in the virus would enable it to spread from human to human?

To find out, Chen's team used plasmid-based reverse genetics to create 127 reassortants, or hybrid viruses, in which they swapped gene segments from H5N1 with those from the H1N1 swine flu virus. In research conducted between January 2010 and December 2011, they infected guinea pigs with the most pathogenic reassortants and found that replacing a single gene was enough to get the virus to leap from infected guinea pigs to healthy ones in adjoining cages.

It was the right experiment for someone whose work ethic borders on masochistic. The 127 hybrid viruses that Chen generated is an "astounding" number, Subbarao says: "That's a tremendous amount of work, and then a tremendous amount of data to stare at and try to see patterns." Completing the study required more than a dozen researchers working for 2 years with more than 250 guinea pigs, 1000 mice, and 27,000 infected chicken eggs. Fouchier says that he has "thought many times of doing the exact same systematic experiment" but did not proceed because of various constraints. Among other issues, "I do not have a single grant for which I could afford to work with 13 people for 2 years to yield a paper," he says.

The virus gain-of-function experiments prompted a global outcry. Earlier studies of this type conducted by Fouchier and Kawaoka in ferrets also sparked an uproar in 2011 when word leaked ahead of publication (*Science*, 2 December 2011, p. 1192), leading to a global moratorium on H5N1 transmissibility studies in January 2012. (The ban was lifted a year later.) In Chen's case, the backlash carried stinging undertones. In comments to British newspaper *The Independent*



Sitting ducks? So far, H7N9 appears to be mysteriously absent in farmed birds.

that were widely reported elsewhere, theoretical ecologist and former U.K. Royal Society President Robert May called the study by Chen and colleagues "appallingly irresponsible," adding that the researchers were "driven by blind ambition with no common sense whatsoever." Volleys came from within China as well. "People are really concerned about the biosecurity" in Chen's lab, says Liu Wenjun, deputy director of the Chinese Academy of Sciences' Key Laboratory of Pathogenic Microbiology & Immunology in Beijing. Liu notes that the deadly severe acute respiratory syndrome virus reemerged in China in 2004, a year after the initial outbreak, when a Chinese CDC worker was infected following a safety breach.

Chen insists that she's unfazed by the criticisms. May is out of his depth, she says: "I don't think he understands this kind of science very well. If a flu scientist said this, I would be concerned." Masato Tashiro, the head of a World Health Organization collaborating center for influenza research in Tokyo, says that he has visited Chen's facility, which includes a biosafety level P3 lab; it meets international standards, he says. The lab is "state of the art," Kawaoka adds.

Some have taken aim at the relevance of Chen's findings. Yi Guan, a virologist at the University of Hong Kong, contends that Chen should have used ferrets, as Fouchier and Kawaoka did. Ferrets better mimic infections in humans, whereas "the guinea pig model has a lot of question marks," Guan says. "You need special lab conditions to make disease happen [in guinea pigs]. I don't think the results are reliable." Chen counters that guinea pigs have both avianlike and humanlike receptors

in the respiratory tracts, which makes them an ideal model for evaluating the transmission potential of viruses that bind to both receptor types.

Chen is now turning her attention to H7N9, which she says appears to infect humans "much, much more easily than H5N1." That naturally raises the question of whether the virus has pandemic potential. But gain-of-function studies are not yet a priority for H7N9, she says. Less is known about this virus than about H5N1, which has circulated for years without acquiring the ability to pass between humans, leading people to ask what changes might make H5N1 transmissible. With

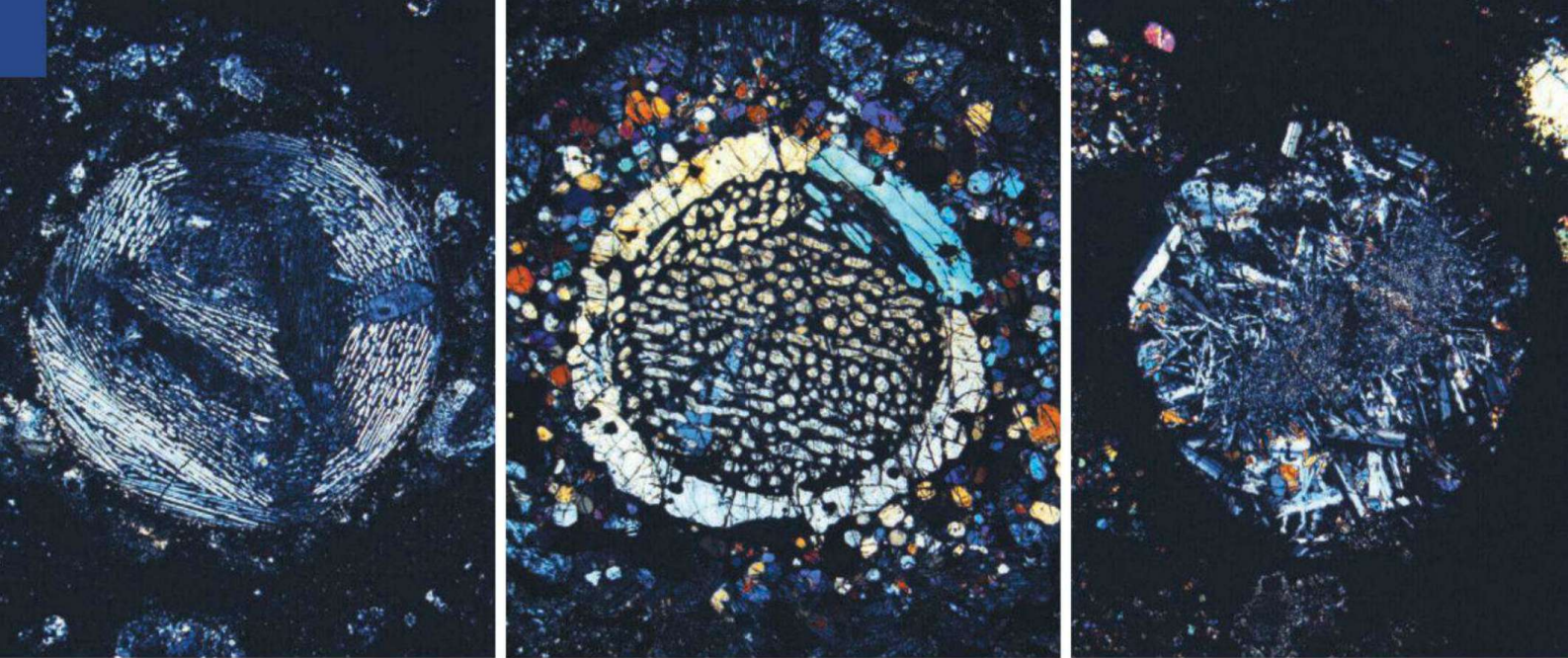
H7N9, Chen says, "first we want to answer the question, *is it transmissible?*"

As Chen dives into H7N9 studies, she may continue to encounter criticism because she is effectively at the helm of China's veterinary research for flu studies. "China is not totally open," says Robert Webster, a virologist at St. Jude Children's Research Hospital in Memphis, Tennessee. When it comes to animal infections, he says, "I'm not convinced that all of the information is completely shared with the world." But Chen is trying, he says: "She does her very best to put out the information that's relevant."

Tens of thousands of tests in China, by the Harbin lab and others, have turned up only one case of H7N9 outside of poultry markets, in a farmed homing pigeon. New infections tapered off in May following the closure of poultry markets, emboldening the Shanghai government to reopen some markets on 20 June. A seasonal lull may have given false reassurance. "H5N1 appeared in the cooler months, disappeared in the hot months, and then reappeared in the cooler months," Webster says. H7N9 may follow a similar pattern, he warns. "Did they know the virus was really gone before they reopened the markets?"

Chen says that she, too, is puzzled by the apparent dearth of H7N9 infections on farms, calling the situation "very strange." If the virus is truly only in poultry markets, she says, "It will be a lucky thing." But she is not the sort to leave matters to chance. Chen says that she had recommended keeping live poultry markets closed permanently. When the mercury starts to fall this winter in Harbin, we'll know who was correct.

—MARA HVISTENDAHL



PLANETARY SCIENCE

Meteorite Mystery Edges Closer to An Answer—Or the End of a Field

Blobs called chondrules in the fabric of rocks from space have long baffled scientists. A new idea may shed light on their origins, but some experts have given up hope

How would you like your decades of research on a field's central problem to be summed up by the statement that "these objects remain as enigmatic as ever"? That was part of the title of a session on the formation of chondrules at the 75th annual Meteoritical Society meeting last year.

For half a century, meteoriticists—scientists who study meteorites—have been trying to understand the origin of chondrules: once-molten, millimeter-size blobs of rock that a 19th century scientist called "drops of fiery rain." Chondrules riddle 85% of the rocks that fall to Earth from the asteroid belt, so meteoriticists are deeply intrigued. And scientists have long presumed that the recipe for making the four rocky planets, including Earth, consisted largely or entirely of chondritic rock. They would like to know how their main ingredient came to be. Yet only two of last year's 14 talks in that chondrule formation session directly addressed the topic, and both of them described a decades-old idea that has made little headway: chondrules splashing off colliding planetesimals.

A dozen years ago John Wood, a leading light of the field, publicly chided his meteoriticist colleagues for their exclusive focus on deciphering the composition of chondrules on ever-finer scales (*Science*,

31 August 2001, p. 1581). That narrow approach "has not worked," he told hundreds of planetary scientists in a provocative 2000 plenary talk, "and it won't work."

What was needed, Wood said the next year, was "a unifying paradigm, and there just isn't one." If chondrules were not to remain "tight-lipped witnesses to the beginning," he said, meteoriticists who know chondrules must collaborate with astrophysicists who know what it was like at the beginning of the solar system.

Wood soon washed his hands of chondrules and retired to concentrate on oil painting, mostly of landscapes, but "his words really resonated with a lot of people," says astrophysicist Steven Desch of Arizona State University, Tempe. Workshops have been held, meteoriticist-astrophysicist collaborations formed, and papers published. "The progress is there," Desch says.

But he emphasizes that it has been slow. Although constraints on proposed formation mechanisms have been tightened and theories have become more sophisticated, the field of possible formation mechanisms has hardly been narrowed in decades. But there may be reason for hope. A collaboration of astrophysicists and a meteoriticist has just floated a new mechanism: humongous "short circuits" in the still-forming solar

system. All it has to do is run the gantlet of skeptical meteoriticists and astrophysicists.

Where's the heat?

Solving the puzzle is hard in part because chondrule formation can't be observed today. The right conditions probably haven't been seen in our planetary system for more than 4.5 billion years. They must have existed during the solar system's first few million years, when only a disk of gas and dust called the protoplanetary nebula swirled around the nascent sun. But meteoriticists have been hard pressed to come up with enough energy even then to rapidly heat rocky dust to 1600 kelvin or more and melt it into globules. The nebula at the time was merely warm—at most several hundred kelvin.

In 2000, meteoriticist Alan Rubin of the University of California, Los Angeles, counted 14 heating mechanisms proposed up to that time. Several involved strong shock waves, from the bow shocks of planetesimals plowing through the nebula to a shock wave trailing off wandering Jupiter. Others ranged from nebular lightning to gamma ray bursts. None, Rubin noted, struck astrophysicists as particularly plausible.

"There are a lot of models out there, but I'm not sure how you can really test them," says Harry McSween of the University of Tennessee, Knoxville. As Wood's first student, McSween did his dissertation work mostly on chondrules but later switched in frustration to studying martian rocks. "However [chondrules] formed, they formed beyond our experience. How do you ever prove it?"

Meanwhile, meteoriticists were generating ever more constraints. By dissecting the chemistry, rock types, and isotopes of chon-

CREDITS: SHERYL SINGERLING/SMITHSONIAN NATIONAL MUSEUM OF NATURAL HISTORY (3)

No peas in a pod. Colorized by mineral type, millimeter-size chondrules from a single meteorite reveal their diversity.

drules and the fine-grained matrix around them down to the micrometer scale, researchers had divided chondritic meteorites—known as chondrites—into four groups and 12 subgroups. The diversity suggested that formation conditions must have varied across the disk without much mixing between formation regions. The rapid heating, it seems, repeatedly struck small parts of the nebula in which concentrations of dust were almost implausibly high. The newborn molten chondrules held on to even their most volatile elements. Some chondrules must have been remelted or partially melted. And the list goes on.

Even in 2001, Wood was losing patience with his colleagues' seemingly endless stamp collecting. "One of the problems with mineralogists and petrologists is we can get bogged down in the details," he said. "I don't think the answer is in the chondrules. They've had the living daylights studied out of them." Meteoriticists have since extracted even more telling details from chondrite meteorites, but to little avail. Some of Rubin's heating mechanisms have since fallen from favor, while remaining ones—including shocks of one sort or another and planetesimal impacts—have a handful of supporters each, and no leading contender has ever emerged.

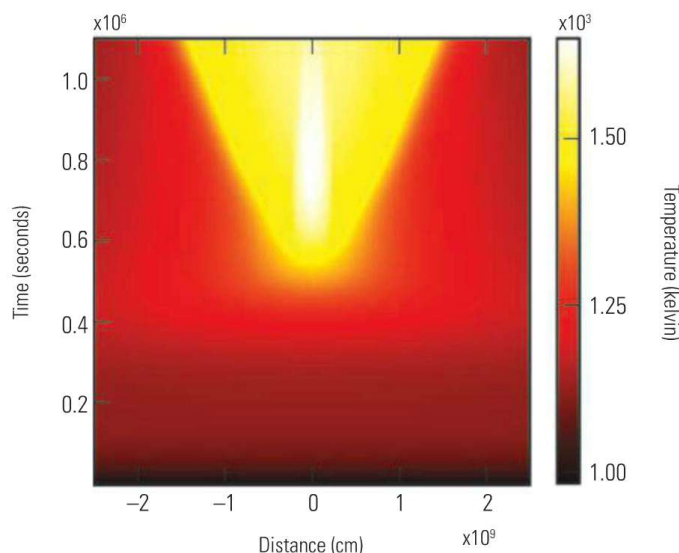
Joining forces

With researchers starting to heed Wood's admonition to cross disciplines, communication between meteoriticists and astrophysicists "is enormously improved over John's day," says meteoriticist Conel Alexander of the Carnegie Institution for Science's Department of Terrestrial Magnetism (DTM) in Washington, D.C.

Two other factors helped as well, Alexander says. Leading astrophysicist Frank Shu of the University of California, Berkeley, made a splash with a proposal that outbursts from the young sun provided the necessary heat (*Science*, 20 June 1997, p. 1789). The idea has not fared too well (none has), but Shu's entry made speculating about chondrule formation "something respectable to do in astrophysics," Alexander says. And the growing fascina-

tion with how planets take shape around other stars "has made people much more interested in what was happening in the early solar system," he says.

The latest product of this growing collaboration comes from four astrophysicists and a meteoriticist. Each of these researchers has or had an affiliation with the American Museum of Natural History (AMNH) in New York City. The astrophysicists of the group were wondering what role the magnetic fields that pervaded the protoplanetary nebula might play in chondrule formation. "Astrophysicists like to see where the energy goes," says astrophysicist Colin



Hot enough. In this simulation of a "short circuit" in the early solar system, temperatures reach rock-melting levels (white) in under a second.

McNally, a former postdoc at AMNH, now at the Niels Bohr Institute in Copenhagen.

So the AMNH researchers followed the well-known energy cascade starting with the gravitational energy stored in the disk's gas and dust orbiting the sun. That energy moved into the magnetic fields churned in the disk by turbulent ionized gas and dust and then to its ultimate dissipation as heat.

Astrophysicists have long known that magnetic field energy can be dissipated as heat through "current sheets." These are relatively thin, spread-out electrical currents spun from the magnetized turbulence of the disk. But normal current sheets would not have been powerful enough to forge chondrules. Last year, though, the AMNH astrophysicists proposed in *The Astrophysical Journal* that the sheets might be prone to a particular instability in which a temperature-sensitive feedback would drive the sheet temperature to rock-melting heights.

Just as an electrical short circuit can surge to wire-melting temperatures when a current finds a path of lower resistance, the new instability would drive up temperatures as atoms of potassium in the nebular gas lose electrons from their shells to become current-carrying potassium ions. The faster the temperature rises, the faster potassium ionizes to carry more current, which in turn raises the temperature even faster. In the AMNH computer simulations published on 20 March in *The Astrophysical Journal Letters*, this runaway current—essentially a short circuit—can raise the core of a current sheet to temperatures ranging from 1650 K

to more than 2000 K, depending on the initial width of the current sheet. So in this model, at least, the proposed instability produces just the range of temperatures that various features of chondrules call for. "It's very natural," McNally says.

Not so fast

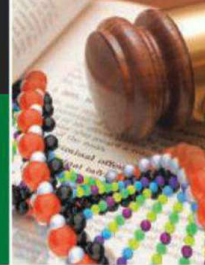
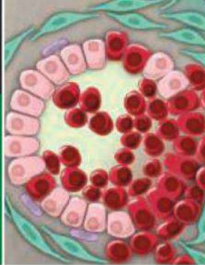
Well, maybe. "It's a new idea, they make a pretty good case for it working, and there's much worth considering," says astrophysicist Alan Boss of DTM, "but I'd put it on the bottom of the list." He doesn't think short-circuits would work in the core of the disk, where most of the mass resides, because temperatures there weren't high enough there to support the instability. Astrophysicist Desch agrees.

Discouraged yet? You have company. Meteoriticist Ian Sanders of Trinity College Dublin has been pushing chondrule formation by impact for 20 years, but he notes that "if you read the literature, none of [the models] work. I sometimes wonder whether there are too many uncertainties and variables."

"We're all a bit disappointed," Alexander allows. What's still needed, Desch says, is better models of chondrule formation. "You have to get your model to the point that you can test it," he says. "You have to make it more quantitative. No one's really doing that."

But Alexander thinks it's going to happen. To start with, McNally has been funded to do such work. And "I do think we're making progress, though perhaps not as fast as we'd like," Alexander says. "People are trying to put chondrites in an astrophysical context," as Wood wanted. "Sooner or later, someone's going to come up with a mechanism that solves it all. I'm an optimist."

—RICHARD A. KERR



LETTERS

edited by Jennifer Sills

Putting Health Science into Health Education

IN HIS NEWS & ANALYSIS STORY “EDUCATORS, LAWMAKERS QUESTION PROPOSED REORGANIZATION” (14 June, p. 1274), J. Mervis discusses our efforts to prevent an end to funding for the Science Education Partnership Award (SEPA) program.

The tiny SEPA program and its smaller siblings at the National Institute of Allergy and Infectious Diseases and the National Institute on Drug Abuse do much more than, as Mervis says, “shar[e] the latest health science results with the public.” In fact, they are the only federal programs that put health science into health education at the K–12 level. In most schools, health education is not taught as part of the science curriculum, and even though standard health education emphasizes behavior modification, the traditional approach has historically failed to affect health literacy: More than 50% of U.S. adults remain functionally health illiterate (1).

One of our SEPA programs (led by K.F.M. and B.J.) illustrates the difference between science-focused and traditional behavior-focused health education. Standard high school health classes about nutrition and obesity focus on behavior modification such as “eat your vegetables,” “eat fewer carbs,” and “eat low fat.” These messages are constantly changing and are often contradictory. As a result, many people (not only students) are confused about what

to believe and end up ignoring guidelines. In contrast, our Metabolic Disease curriculum asks the question, “What information do I need to make a judgment about which nutritional advice I should follow?” We teach 10th- to 12th-grade students how to distinguish correlative from causative information, identify what criteria a study should meet to produce reliable information, and determine whether a comparison between two different studies is valid. Our goal is to give students tools they can use to evaluate the science underlying health information, as opposed to merely giving them the information itself. Another of our SEPA programs (led by J.M.W.) teaches high school students about genetic and environmental factors that influence complex diseases like diabetes, hypertension, and cancers, subjects that are never addressed in traditional health education. A nuanced understanding of these topics will become increasingly crucial in the

coming decades as the fruits of the National Institutes of Health (NIH) investment in genomic sequencing are finally harvested.

It is therefore troubling that NIH elected to eliminate funding for these programs entirely rather than subject them to cuts, even though neither the continuing resolution nor the sequester pressured them to do so. Why? In the News story, NIH Deputy Director Lawrence Tabak says that NIH plans to provide only technical expertise after the reorganization, implying that NIH’s higher administration believes that NIH has no responsibility to fund K–12 health education that is firmly rooted in science. This seems disingenuous given the NIH mission to “apply knowledge about the nature and behavior of living systems to enhance health, lengthen life, and reduce the burdens of illness and disability” (2). Preparing STEM workers for the workforce and educating the U.S. citizenry in health literacy are different tasks that were clearly

erroneously conflated by the STEM consolidation committee. In contrast, health science is legitimately intertwined with health science literacy in the 21st century. The agency responsible for health science should also be responsible for health science education.

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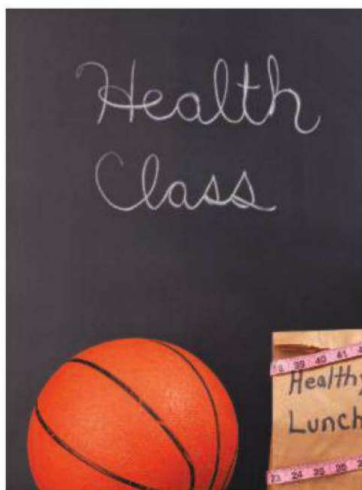
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2. NIH, Mission (www.nih.gov/about/mission.htm).

The Value of Incentives in Blood Donation

IN THEIR POLICY FORUM “ECONOMIC REWARDS to motivate blood donation” (24 May, p. 927), N. Lacetera *et al.* discuss evidence that paying donors for blood may increase donations. Here, I make two clarifications and explain why I have concerns with excessive incentives or cash payments offered to blood donors.

First, the U.S. Food and Drug Administration (FDA) does not technically prohibit monetary (cash) payments for blood donation, but it does require that blood and blood components obtained from donors given cash or incentives “readily convertible” to cash be labeled as being from paid donors. (1).

Second, Lacetera *et al.* reference *Flynn v. Holder*, a recent U.S. Court of Appeals decision concerning a statute that prohibits monetary compensation to bone marrow donors (2). The *Flynn* decision itself, which allowed compensation for peripheral blood stem cells, cannot accurately be interpreted to apply to blood donation, but the general idea of giving cash payments to blood donors may,

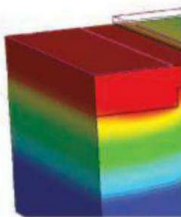


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How the neck
evolved

139



Wrap-around
transistors

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unfortunately, be reconsidered as a result of the court's ruling.

As Lacetera *et al.* point out, as recently as the 1970s, many U.S. blood donors were paid in cash or, even more recently, given "credit" to reduce costs should they or a family member ever need blood (3, 4). In their 1978 paper, Surgenor and Cerveney described Blood Services of New Mexico's (BSNM's) transition in 1972 to 1974 from using paid to volunteer donors. They wrote that "[t]he abandonment of paying donors in cash...was in reality the abandonment of BSNM's dealing with individuals. Instead BSNM began to relate to the community at large. Everyone was a potential donor."

Blood donation is as much an expression of community altruism as of individual generosity. Excessive nonmonetary incentives to donors and cash payments may undermine the perception of blood donation as being special and the community spirit that has encouraged so many to donate day-to-day and during emergencies (5). Accordingly, some blood establishments should be more attentive to the value of incentives offered to their volunteer donor base.

MITCHELL BERGER

Center for Biologics Evaluation and Research, Office of Blood Research and Review, U.S. Food and Drug Administration, Rockville, MD 20852, USA, November 2008 to April 2013. Present address: Exton, PA 19341, USA. E-mail: mazruia@hotmail.com

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2. *Flynn v. Holder* (U.S. Court of Appeals for the 9th Circuit, No. 10-55643, 1 December, 2011).
3. Food and Drug Administration, Center for Biologics Evaluation and Research, Workshop: Recruiting Blood Donors—Successful Practices, Bethesda, MD, 6 to 7 July 2000; www.fda.gov/downloads/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/TranscriptsMinutes/UCM055391.pdf.
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Response

THE GOAL OF OUR POLICY FORUM WAS TO present evidence from large-scale observational studies and field experiments that—across countries, many distinct reward items, and individuals who had and had not donated before—economic incentives increase blood donations (1–6) without affecting safety (1, 3, 5). Berger points out that the FDA does not prohibit monetary compensation for blood donors. However, as we stressed in our Policy Forum, many blood collection agencies follow World Health Organization guidelines that promote the position that blood should be obtained from unpaid volunteers only. Even when there are no legal restrictions (7), organizations such as the American Red Cross Blood Services do not collect blood from donors paid in cash (8). We also noted that the evidence comes from gift items not explicitly framed as payment, and we stressed caution in extrapolating to cash payments.

Berger further argues that larger non-monetary gifts undermine the altruism behind giving blood. We are unaware of any study examining whether large gift incentives corrode the moral value of donating blood. The

evidence that we reviewed, however, shows the largest positive effects on donations from the highest-valued incentives (including the paid day off work which has existed in Italy for over 40 years).

Berger also questions the applicability of the *Flynn v. Holder* case to the issue of blood donation. Beyond the decision's legal ramifications, *Flynn v. Holder* has ethical implications for blood donation (9). One reason the case was brought to the courts was to decide whether someone could offer rewards to obtain bone marrow to save a child's life. If evidence emerges that economic rewards corrode moral value for donating blood, it remains to be determined whether this ethical cost outweighs the benefits of greater supply.

How blood donations should be perceived and which ethical principles should guide society are critical questions. Citizens and policy-makers should make these decisions; we believe that implications for patients should also be considered, and empirical evidence should play an important role in the process.

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CORRECTIONS AND CLARIFICATIONS

News of the Week: "Ocean models help swimmer navigate Florida Straits" (21 June, p. 1386). Villy Kourafalou is an oceanographer who leads the Coastal Modeling Group, not a meteorologist. Kourafalou's group provided the high-resolution model currents and ROFFS streaming satellite data of surface ocean conditions. The HTML and PDF versions online have been corrected.

Editors' Choice: "Hop to evolution" by L. M. Zahn (7 June, p. 1142). The colloquial name for the insects described was misstated. The common name for insects in the *Oliarus polyphemus* species complex is leafhoppers, not cave hoppers. This has been corrected in the HTML and PDF versions online.

News Focus: "Astronomers lend know-how to cleanup" by D. Normile (1 March p. 1029). The article incorrectly states that the energies of the gamma rays emitted by cesium-137 and cesium-134 are higher than what the Soft Gamma-ray Detector (SGD) is designed for. The SGD's range does cover the gamma rays emitted by cesium. However, the SGD was still under development at the time of the accident, so the Institute of Space and Astronautical Science team modified a different ASTRO-H instrument so it could observe the cesium gamma rays using the detection principle of the SGD. The PDF and HTML versions online have been corrected.

Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

EVOLUTION

Adaptation, Fast and Endless

Jordi Bascompte

Evolution shapes life on Earth tirelessly. This statement may sound somewhat trivial, but when supported by so many recent and diverse studies it becomes exciting and timely. This is clearly demonstrated by John Thompson's *Relentless Evolution*. His lucid and extensively referenced synthesis also shows why understanding evolution matters—especially today, a time of rapid environmental change.

The book's contents will strike many readers as novel. That is because in the past few years we have largely changed our views about the tempo and nature of adaptive evolution. For example, whereas a couple of decades ago almost everyone would claim

long argued for the crucial role the evolution of interactions among species plays in generating biodiversity [e.g., (1–3)]. As he notes in this book, researchers in the 20th century stressed the evolution of species, but those of the current century will emphasize the evolution of interactions. We know that many fast-evolving parts of genomes are involved in the interactions with other species. Recent years have seen a progression in changing the focus of this overall claim from pairwise interactions, to small groups of species interacting in geographic mosaics, to

large networks of dependencies among free-living species. Thus, adaptive evolution often leads to complex networks of interacting species that in turn spur additional selective pressures. This is one reason for the never-ending character of evolution.

Another key component in our appreciation that adaptive evolution is so pervasive and fast relies on the fact that the direction of evolution may change markedly through time and space. The wonderful work by Rosemary and Peter Grant with Darwin's finches (*Geospiza*) in the Galápagos Islands offers a

great account of the former. The alternation of dry and wet years has been shown to be enough to reshape the selective forces on the finches' beak size. During a major drought, up to 85% of the medium ground finches on the island of Daphne Major died. The surviving individuals were much larger, with stronger beaks able to crack open the strong seeds of the then-dominant plant species. This trend was reversed during more humid periods, which had a greater diversity of plants, some of them with much weaker seeds. An anecdotal but insightful indication of the speed of adaptive evolution: Thompson notes that when he contacted the Grants for permission to use a modified version of a graph they had

published a few years earlier (which showed the evolution of beak size in one of Darwin's finches over 30 years), they told him about an updated version that showed another shift in the direction of change. Thompson felt he was racing to produce a timely account of our current understanding of adaptive evolution. Is there still anyone who thinks that evolution always proceeds slowly?

Similarly, we now understand that populations are not well mixed among their habitats, but often occupy discrete habitat patches in heterogeneous environments. Thompson's classic work on the geographic mosaic theory of coevolution provides a wonderful illustration. Examples range from his own research with *Greya* moths and woodland star (*Lithophragma* species) plants to the coevolution between red crossbills (*Loxia curvirostra*) and conifers by Craig Benkman and colleagues. Rather than an environment in which there is a single evolutionary process, we find in such cases a collection of seemingly independent evolutionary processes among heterogeneous environments. This is yet another reason why evolution is so tireless and capable of producing so many diverse adaptive solutions.

Thompson is an authoritative writer. As one also finds with the late ecologist Ramon Margalef, his books are linked one to another by the personal evolution of a thinker focusing on a major subject, in this case the evolutionary and coevolutionary mechanisms that shape biodiversity. That is, his latest book addresses much the same major questions as its predecessors, and yet it is timely and distinctive because Thompson's way of thinking, as with the subject of his analysis, evolves and diversifies rapidly through time. The book continues an unfolding story that becomes richer and more appealing as more evidence is compiled. Thompson's discussions confirm previous ideas but at the same time channel them toward novel and richer directions. They will also serve to remind young scientists that this is a great time to be conducting research on evolution.

Timely, authoritative, and beautifully told, *Relentless Evolution* is a must read for anyone interested in understanding the processes shaping life on Earth. As Thompson reminds readers in the concluding chapter, the big challenge we face is not simply the loss of species but the consequent loss of evolutionary history and potential. "We still have much to learn about ... how we can use our developing knowledge of the relentlessness of

Relentless Evolution

by John N. Thompson

University of Chicago Press,
Chicago, 2013. 509 pp. \$100,
£70. ISBN 9780226018614.
Paper, \$35, £24.50. ISBN
9780226018751.



Different moths, different shapes. Subspecies of Joshua trees (*Yucca brevifolia*) pollinated by *Tegeticula synthetica* moths (left) and by *T. antithetica* (right).

that ecological and evolutionary time scales were uncoupled, we now know that evolution can proceed very rapidly and therefore that ecology and evolution should be studied jointly. Similarly, although the focus of much earlier evolutionary research emphasized the adaptation of species to their environments, we now understand the major importance of adaptation to other free-living species in generating diversification.

Thompson (an evolutionary ecologist at the University of California, Santa Cruz) has

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evolution to maintain the diverse web of life and our place within that web.”

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10.1126/science.1239702

SCIENCE AND CULTURE

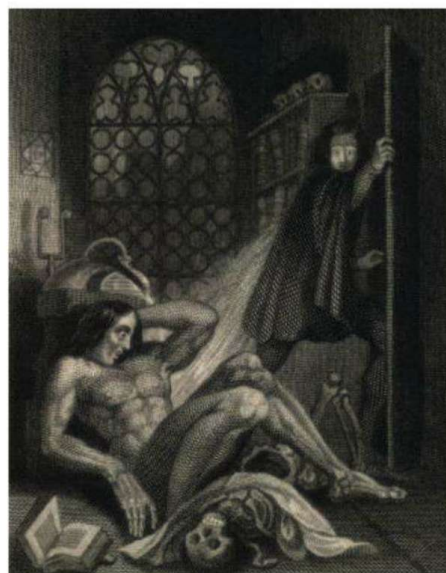
Origins of the Modern Prometheus

Michael A. Goldman

As scientists, we have a turbulent relationship with science fiction, not to mention the broader poetry and literature and those who practice in these dark arts. This is especially so when writers warn us of the arrogance and the hazards of our own work. But from the mid-18th century into the first decades of the 19th, England's high society, literary society, and scientific innovators shared the parlor. In *The Lady and Her Monsters*, Roseanne Montillo transports the reader into that dark and stormy, intensely curious, romantic, and macabre slice of history. Her sweeping biography of Mary Wollstonecraft Shelley portrays a time that saw the poetry of Keats and Byron; the dawn of a new, experimental physiology and medicine; and the horror of *Frankenstein; or, The Modern Prometheus* (1).

The daughter of William Godwin and Mary Wollstonecraft, Mary Shelley would be surrounded by death, intrigue, and storm from the start. The night of her birth, London experienced a storm “later remembered as one of the most awesome displays of thunder and lightning anyone had ever seen.” While natural philosophers were learning to master electricity, others saw their efforts as sacrilegious and the angry thunderstorms a sign of God's wrath. The worst was still to come: Wollstonecraft, a writer and philosopher whose *A Vindication of the Rights of Woman* is often credited as the first feminist tome, died 11 days later from an infection acquired during childbirth. Now there's a memorable entry into the world.

After the family moved for a new start, their home was near the city's prisons and the



frequent hangings that brought Londoners into a frenzy. More positively, Godwin hosted gatherings of many thinkers of the day, such as Samuel Taylor Coleridge, who read his *The Rime of the Ancient Mariner* at one. Montillo (who teaches literature at Emerson College) notes, “A decade later Mary Godwin would use similar imagery in the opening scenes of ... *Frankenstein*. ... In it, the fictional character of Robert Walton, a mariner and explorer intent on finding a passage to the North Pole, appears and echoes Coleridge's mariner as he too was traveling into uncharted waters.”

Conversation in the Godwin home certainly included the concept of animal electricity, which had been around since at least the mid-1700s. The ideas of electricity's role in physiology, its curative properties, and the potential for reanimation of dead tissue had, by Mary Godwin's youth, “become fashionable in all of European society”—from the natural philosophers to “more amateurish individuals” and also “among artists and writers and at crowd-pleasing soirées and salons all over England, France, and Germany.” Poet Percy Shelley was among those who experimented with this “vital force existing in humans and nature” and later wrote “long poems and odes that mused on the sublime mysteries of the natural world and the awesome powers of lightning and thunder.”

Later, Mary Shelley's travels with Percy would lead them to the town of Nieder-Beerbach, where they would see Burg Frankenstein. Percy and Mary collected local folk tales, and it is possible that they heard much

The Creature. Theodor von Holst's frontispiece to Mary Shelley's 1831 edition.

about the rich histories of the Frankenstein castle and the family for which it was named. Montillo presents Radu Florescu's controversial claims (2) of a link between Mary's novel and the alchemist Johann Konrad Dippel (1673–1734). Dippel, born at Burg Frankenstein, became the stuff of local legends: He was believed to have found the philosopher's stone (able to prolong life and turn lead into gold). But his use of the stone for his own gain, and his lack of knowledge of chemistry, resulted in his burning down the place. Parallels with the characters, events, and scenes in the novel are palpable.

Montillo's account is certainly not a spoiler for the novel or the many plays and movies that followed. She reminds us that the blockbuster 1931 film from Universal Studios is a work of pure entertainment that “turned Mary Shelley's moral tale into a basic horror story,” ignoring its most important messages.

Science fiction is valued not just for its entertainment value or the cautionary message it sends, but because the popular literature—especially when it erupts into theaters and broader discussion—provides an excuse for scientists to teach, and the general public to learn,

about the work scientists do. *Frankenstein* is almost 200 years old, but its message today (with few exceptions) remains the same. Mary Shelley's book is a must read. Montillo's biography, though not quite the “science of *Frankenstein*” I had hoped, provides a rewarding addition of scientific and social context. With a time sequence that is hard to follow and two Marys whose lives are rife with romantic intrigue, tragedy, death, and depression, *The Lady and Her Monsters* isn't an easy read. The story of Mary Shelley's creation, life, and death mirrors the story of Frankenstein and is almost as entertaining and enlightening as the monster's tale. Addressing humanity's coping with its newfound powers, they are stories for every age.

The Lady and Her Monsters

A Tale of Dissections, Real-Life Dr. Frankensteins, and the Creation of Mary Shelley's Masterpiece

by Roseanne Montillo

William Morrow (HarperCollins), New York, 2013. 332 pp. \$26.99, £17.30. ISBN 9780062025814.

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10.1126/science.1240948

Want to win a rather special prize in Stockholm, Sweden this December?



Henrik Rygg / Image bank, Sweden

Winner's essay published in the journal *Science*
\$25,000 dollars grand prize
Awards held in Stockholm in December



This December a rather special prize will be awarded in Stockholm, Sweden. The journal *Science* and SciLifeLab have come together to recognize and celebrate excellence in PhD research. The *Science* and SciLifeLab Prize has been established to support young scientists at the start of their career.

“Science has never been more exciting and, as leaders in science, we need to support and encourage young researchers today and tomorrow. This prize is a way of doing just that.”

Professor Mathias Uhlen, Director SciLifeLab

The grand prize winner of this major global award will have their essay published in the journal *Science* and receive \$25,000. Three runners up will receive a combined total of an additional \$10,000 in prize money.

The prizes will be presented in Stockholm, Sweden in the middle of December 2013.

To enter

You must be a recent Ph.D. graduate (awarded between January 1, 2011 and December 31, 2012).

Submissions must be in the form of a 1000 word essay, in English, on your thesis, highlighting the significance of its contribution and overall implications in the field. The four submission areas for this prize are:

- (1) Genomics / Proteomics / Systems Biology
- (2) Developmental Biology
- (3) Molecular and Cellular Biology
- (4) Environmental Life Science.

The deadline for submissions is August 15, 2013. The overall winning essay will be published in *Science*.

For further details and to enter, please go to: www.sciencemag.org/scilifelabprize

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SCIENCE AND LAW

Revising China's Environmental Law

Guizhen He,¹ Lei Zhang,^{2,3} Arthur P. J. Mol,^{2,3} Yonglong Lu,^{1*} Jianguo Liu⁴

China's Environmental Protection Law (EPL) is the main national environmental legislative framework. Yet the environmental legal system is incomplete, and implementation and enforcement of environmental laws have shown major shortcomings (1–3). A controversial attempt to revise the EPL could have far-reaching impacts on China's economic development and environmental protection, which may have global implications (4, 5). Increasing pressures to strengthen the rule of law in China raise the stakes (6). We discuss the need for a sound legal and scientific basis for revising the EPL.

The National People's Congress (NPC) (the highest legislative body in China) Standing Committee included major revision of the EPL in its 2011 legislative agenda [see supplementary materials (SM)]. Proponents for radical revisions [e.g., the Ministry of Environmental Protection (MEP)] opposed other agencies and ministries that favor incremental adjustments. The 2012 draft revision shows the power of the incrementalists: Only the most urgent, feasible, and commonly agreed-upon improvements that require little change of other existing environmental laws have been included (7, 8). Release of the draft revised EPL has triggered a flood of questions, comments, and complaints (4, 5, 8) (SM).

Debates resounded during the 2012 National Congress of the Communist Party of China (CPC), when top leadership changed and “Ecological Civilization” (restructuring the economy to achieve man-nature, production-consumption harmony) was included in the Constitution of the CPC, with emphasis on scientific and democratic governance under the rule of law (9, 10). Inclusion in the Constitution strengthened the legal and authoritative position of ecological civilization in development planning.

Because EPL revision was not approved by the NPC in March 2013, a new round of drafting is in process. The Legislative Affairs Commission of NPC has listed an EPL revision in the 2013 legislation plan. We suggest addressing the following four major issues.

In compliance with the Constitution, environmental protection and ecological civilization as national basic policy must be reaffirmed. The EPL should provide a legal basis for key environmental principles: the precautionary and prevention principles, public environmental rights and participation, and environmental justice (11, 12). These are absent or insufficiently stressed in the current draft.

A strong legal basis must be provided for independent strategic environmental assessment and performance-based auditing. The current Environmental Impact Assessment (EIA) law only requires EIA of plans or projects not of policies (13). Although after-the-fact environmental audits should be conducted on all major public projects and programs by independent auditing institutions, few have been conducted because of limited capacity and knowledge within the National Audit Office and lack of legal backup (14). Environmental audits should be indispensable parts of decision-making of major governmental investments. EPL revision provides an opportunity to remove obstacles for powerful policy and to plan EIAs and governmental environmental audits crucial for science-based environmental policies.

Law enforcement must be improved. Principles for defining, coordinating, and supervising transregional and inter- and intra-departmental environmental rights, responsibilities, and obligations of governmental and nongovernmental actors need to be specified in the revised EPL. Internal and external evaluation of environmental performance of governmental organizations and officials should become compulsory and transparent (12, 15). Adequate rules for punishment must be set up and enforced to penalize those who violate the law—administrators, regulators, and regulated parties alike (1), e.g., through double punishment (punish the violating company and its owner), a daily penalty for continuous environmental violations, and avoiding low penalties. To align with litigation laws, the revised EPL should adopt public interest litigation and grant any public entity or citizen the right to bring violating administrative departments and other entities to court (5, 7).

The revised EPL should shift from regulation to governance, promoting participation of nongovernmental stakeholders and balancing “hard” instruments (e.g., command-

The law must affirm basic principles, strengthen assessment, improve enforcement, and enhance governance.

and-control) and “soft” (e.g., environmental education and voluntary agreements) (6, 16, 17). More transparency and public participation in policy and regulatory processes at all stages, from drafting legislation to enforcement activities, can improve policy effectiveness and address potential inconsistencies. Revision of the EPL can improve the government's legitimacy for promoting ecological civilization by following expert advice, including public suggestions, and empowering environmental authorities for sustainability (18). It is a unique opportunity for China to be a role model, especially for other emerging economies.

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Acknowledgments: This work was funded by the National Natural Science Foundation of China (71103175 and 41071355), International Science and Technology Cooperation Program of China (2012DFA91150), the Netherlands Royal Academy of Arts and Sciences, and the Chinese Academy of Sciences (11CDP028), Ministry of Education of China (NCET 10-0806), U.S. NSF, and AgBioResearch of Michigan State University. We thank R. Stone for comments.

Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6142/1233/DC1

10.1126/science.1235000

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CANCER

Prostate Cancer Takes Nerve

John T. Isaacs

One in six American males will develop prostate cancer during their lifetime, and once prostate cancer metastasizes to distant sites, it is incurable at present (1). This translates into the sobering reality that more than a quarter of a million men will die of prostate cancer this year throughout the world (2). Thus, new therapeutic approaches to this devastating disease are urgently needed. In this regard, preventing and/or disrupting neoneurogenesis—the ingrowth of nerve endings into the tumor—in prostate cancer is a newly discovered therapeutic target. Innervation of the prostate gland controls its growth and maintenance, but little is known about the function of neurons in prostate cancer. On page 143 of this issue, Magnon *et al.* (3) report how the autonomous nervous system (which controls internal organs and glands, below the level of consciousness) stimulates the initiation and metastasis of prostate cancer.

The human prostate is a stratified tubule-alveolar gland composed of a well-developed stromal compartment containing fibroblasts, endothelial cells, nerves, and abundant smooth muscle cells. The stroma surrounds an epithelial compartment containing a continuous layer of basal cells upon which rest a layer of luminal epithelial secretory cells (see the figure). The prostate is one of the male sex accessory sex tissues, which provide the fluid components of the ejaculate.

The prostate gland receives both sympathetic and parasympathetic neural input. Sympathetic fibers functionally innervate the stromal smooth muscle cells; parasympathetic fibers innervate the epithelium. Thus, sympathomimetic drugs induce expulsion of prostate secretion through contraction of stromal smooth muscle. These drugs stimulate the release of noradrenaline from adrenergic fibers, which then binds to and activates β -adrenergic receptors on muscle cells. Parasympathomimetic drugs stimulate cholinergic fibers to release acetylcholine, which binds to muscarinic receptors on epithelial cells and triggers their tonic secretory output.

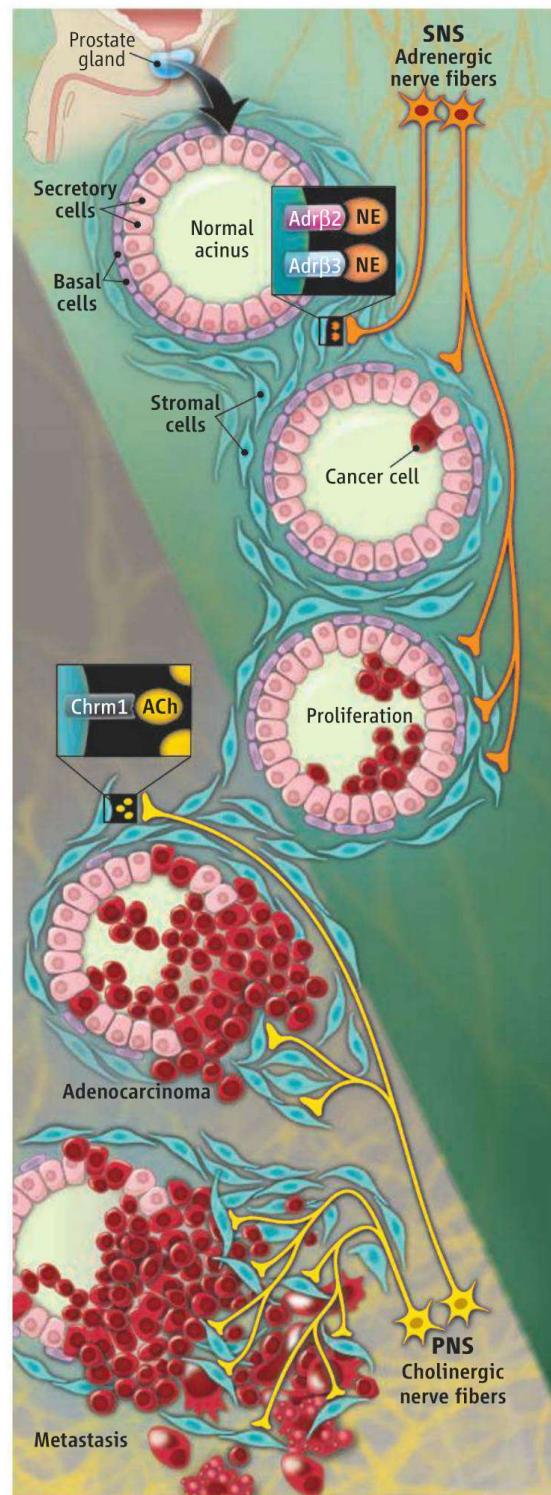
Until quite recently, the role of the sympathetic and parasympathetic nerves in the

development and progression of prostate cancer was underappreciated even through it was well established that perineural invasion by prostate cancer cells (cancer cells track along a nerve fiber within the prostate, spreading to spaces surrounding the nerves and outside the prostate) correlated with poor prognosis and that nerve density is increased in cancerous areas of the prostate as well as preneoplastic prostate lesions (4). On the basis of preclinical mechanistic studies, as well as pathological correlations, it was proposed that analogous to neoangiogenesis, in which cancer cells release factors that elicit the growth of blood vessels into the tumor, prostate cancer cell interaction with nerves induces a neoneurogenesis response that promotes both neurite outgrowth and axonogenesis into the tumor (4). Using a mouse model of prostate cancer, it was shown that enhancing epithelial expression of transforming growth factor- β 1, which is characteristically overexpressed in human prostate cancer, induces inflammation of nerve ganglia within the prostate (5). These observations suggested that cancer associated neoneurogenesis could be a “wound-healing” response to such inflammation. Because inflammation is a characteristic “driver” of prostatic carcinogenesis, this concept was reasonable (6).

Magnon *et al.* used a series of human prostate xenograft and transgenic mouse models in combination with pharmacologic agents to document that the neoneurogenic response induced in sites of prostate cancer involves sympathetic and parasympathetic nerves. The authors observed that the neoneurogenic response stimulates the initial phase of prostate cancer development through adrenergic fibers from the sympathetic nervous system (SNS). The release of noradrenaline stimulates β_2 - and β_3 -adrenergic receptors expressed on smooth muscle cells in the stroma. Cholinergic fibers of the parasympathetic nervous system (PNS) stimulate invasion, migration, and metastasis of prostate cancer cells by releasing acetylcholine that stimulates Chrm1 muscarinic receptors on stromal cells (including fibroblasts and smooth muscle cells).

The findings of Magnon *et al.* “credential” the neoneurogenic process as a highly relevant therapeutic target for both the prevention and treatment of prostate cancer.

Prostate cancer development and metastasis is driven by invasion of the tumor by the nervous system.



Tumor innervation. Prostate cancer is initiated by sympathetic nerves that release norepinephrine (NE), which activates adrenergic receptors (Adr β 2, Adr β 3) on stromal cells. Parasympathetic nerves release acetylcholine (ACh), which acts on type 1 muscarinic receptors on stromal cells, promoting cancer cell proliferation and metastasis.

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SCIENCE

Additional support for the qualification of neoneurogenesis as a new therapeutic target is provided by epidemiologic studies documenting that prostate cancer patients taking adrenergic β -blockers have a lower mortality (7). These important results require follow-up with appropriate preclinical mechanistic studies—it is not clear how neurostimulated stroma supports the different stages

of prostate cancer progression and spread. These insights should help not only to identify the most appropriate drugs for clinical testing, but to determine the optimal timing to prevent and/or disrupt these neoneurogenic pathways in prostate cancer.

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10.1126/science.1241776

CHEMISTRY

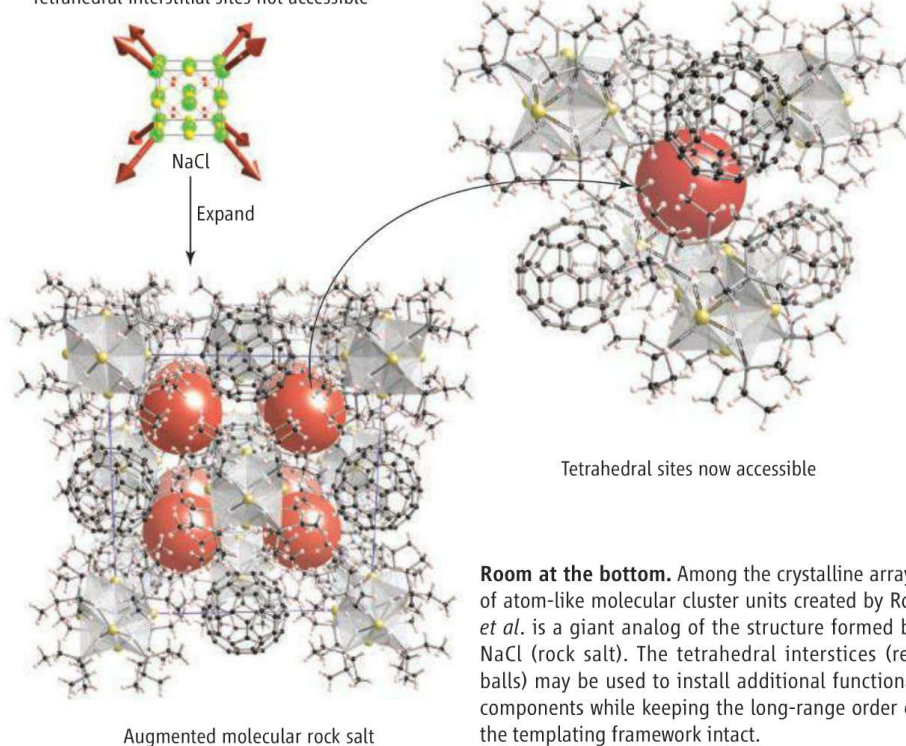
Tuning Molecular Solids

Patrick Batail

Molecular clusters can behave in a way similar to elemental atoms, for example forming solid assemblies with the same structures as rock salt (see the figure), cesium chloride, or molecular perovskites. On page 157 of this issue, Roy *et al.* (1) report molecular binary solids that are expanded analogs of rock salt or cadmium iodide, with inorganic clusters and C_{60} occupying the atomic sites. The use of two different molecular clusters, one of which is electron-poor, the other electron-rich, distinguishes the work from earlier studies (2, 3). Modification of the outer ligands on the components may allow the electronic properties of the material to be changed while maintaining the overall structure.

The structure of molecular solids results from a delicate balance between several forces, including overlap interactions between π -type orbitals, hydrogen and halogen bonds, and electrostatic and van der Waals dispersion forces. However, the bonding interactions that determine the structure are not necessarily those that determine the electronic properties (4). The balance between hydrogen and halogen bonds and electrostatic and van der Waals forces are essential to direct the structure, yet these interactions do not contribute to the dispersion of π -type bands at the Fermi level that determine the conductivity of molecular solids. The simple structures reported by Roy *et al.* provide a basic framework to determine the electronic structure and to analyze the overlap of the π -type orbitals of the cluster units. This is important for understanding how the electronic structure may change when the molecular units are modified.

Tetrahedral interstitial sites not accessible



Molecular clusters and fullerenes form ordered structures with potentially tunable properties.

Room at the bottom. Among the crystalline arrays of atom-like molecular cluster units created by Roy *et al.* is a giant analog of the structure formed by NaCl (rock salt). The tetrahedral interstices (red balls) may be used to install additional functional components while keeping the long-range order of the templating framework intact.

Another essential feature of Roy *et al.*'s work relates to the degree of charge transfer between the cluster units in the solid state (5). The structures are made from assemblies of charge-rich and charge-poor molecular precursors. In the solid state, charge is transferred between the components, with a net reduction of C_{60} by one electron. The charges remain localized in the lattice, but addition of π -functional ligands to the cluster units may increase the overlap between the clusters' orbitals so that they will spread into bands, which lead to long-range electron delocalization and metal-like conductivity.

Furthermore, similar to the superconductors series A_3C_{60} (3), the structures contain

tetrahedral interstitial sites that may allow insertion of additional components (see the figure). Roy *et al.*'s molecular rock-salt structure, thus, has the potential to keep its integrity while serving as a template for hosting smart components. Insertion of additional molecular components in some or all tetrahedral sites will increase the complexity of the system (6), but it will remain straightforward to decipher this complexity in a rock-salt framework (7). Deliberate manipulation of molecular shape, frontier orbitals, redox interactions, electron transfer, or insertion of molecules that can rotate (8, 9) may result in multifunctional materials that combine properties such as conductivity, magnetism, and ferroelectricity.

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Likewise, further developments may be envisioned by using C_{60} derivatives. One exciting possibility would be to use the prototypical electron acceptor of bulk-heterojunction polymer cells, PCBM ([6,6]-phenyl- C_{60} -butyric acid methyl ester), on account of its solubility and ability to crystallize. Installing PCBM in C_{60} octahedral sites in Roy *et al.*'s expanded rock-salt structure and also using the tetrahedral sites for further tuning would provide a model for the kind of molecular assemblies that are thought to be responsible for high coherent carrier transport in crystalline solar cells (10, 11).

A major hurdle in the development of functional molecular materials has been the lack of predictability of the structure of assemblies held together by weak noncovalent interactions. This is especially important given the synthetic challenges encountered in the preparation of the molecular precursors. The work by Roy *et al.* is an exciting and seminal step toward filling this gap. Deliberate tuning of these structural prototypes is likely to lead to new materials with exciting properties.

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10.1126/science.1240813

MATERIALS SCIENCE

Nature's Other Self-Assemblers

William E. Bentley and Gregory F. Payne

There is a continuing quest to precisely fabricate soft matter for emerging opportunities in the medical and life sciences. Often this quest looks to nature as a source of materials or inspiration, and often the journey leads to polypeptides, nucleic acids, or their mimics. On page 154 of this issue, Ejima *et al.* (1) look elsewhere to find another self-assembling biological material—a search based on phenolics.

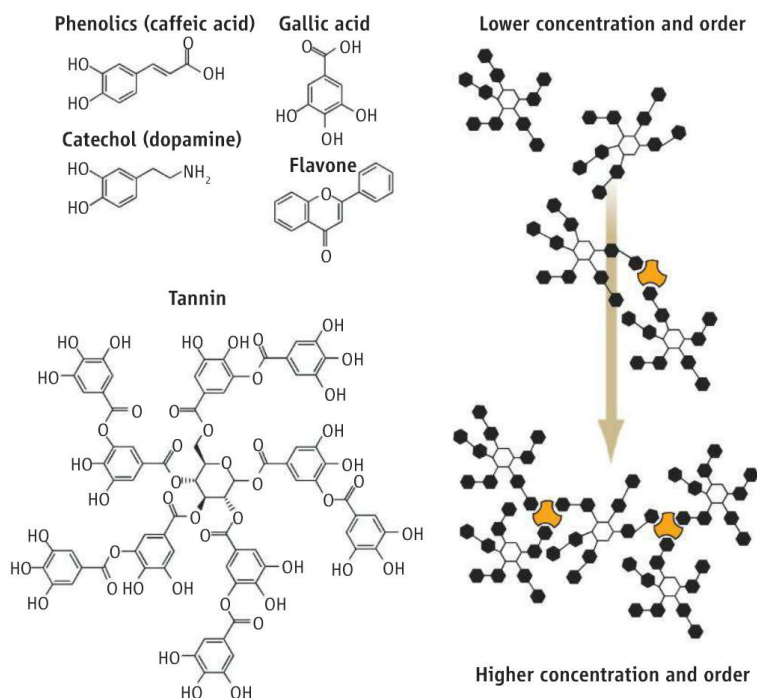
Phenolics are ubiquitous. More abundant than proteins or nucleic acids, they include the humics in soil, the lignin in trees, and the melanin in skin and hair. Small-molecule phenolics are also abundant, offering a range of properties and an array of (putative) functions: Antioxidant phenols in our diet promote health; salicylic acid and catecholamines, respectively, mediate cell-cell communication among plant cells and between neurons; and phenolics provide cross-linkers for insects to harden their cuticles. Despite their abundance and the simplicity of the phenolic, catecholic, gallic, or flavonoid building blocks,

phenolic materials remain a mystery (see the figure). Phenolic moieties are weakly acidic can donate an electron or electron pair, form reactive intermediates, undergo radical or electrophilic reactions, chelate metals, bind to surfaces, and form π stacks (2). Although these diverse capabilities make phenolics versatile, they also tend to make them intractable, especially with respect to understanding their hierarchical and supramolecular organization. Forget the big questions of melanin's physiological and pathological activity; the

Materials based on phenolics may provide another natural route to fabricating exotic structures with advanced functionality.

field is still debating whether its structure is that of a polymer or an aggregate (3).

But from this chaos emerges simplicity. The tannins investigated by Ejima *et al.* bind to surfaces and are cross-linked by coordination with iron (Fe^{III}). This self-assembly process is simple, occurring in a single step; it is fast, occurring in minutes; and it is reversible by altering the pH. The process also appears to be generic, in the sense that the surface doesn't seem to matter. The surface can be removed after the Fe^{III} -tannin film is



Brought to order. Phenolics are diverse and ubiquitous in nature. Tannins, which have traditionally been used for leather tanning, are now reported by Ejima *et al.* to self-assemble with iron to generate thin films.

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formed (a capsule can be generated by self-assembling Fe^{III}-tannin onto a sacrificial template). This approach is reminiscent of earlier work (4) showing that another phenolic, dopamine, could assemble onto surfaces and then undergo subsequent reactions to provide a simple, versatile, and generic coating, even if the “polydopamine” name may need to be revisited because it too may be an aggregate instead (5). The approach also harkens back to even earlier work (6) that showed the critical role of dopamine residues in the adhesion of mussel glue protein. Despite the technological precedents, the report of Fe^{III}-tannin films may mark a beginning, as these films may be capable of more than self-assembly.

Before the focus on adhesives and self-assembly, the possibility was raised that melanins possess electronic properties (7). More recent studies indicate that the ability of lignins to undergo redox cycling enables them to perform capacitor functions of accepting, storing, and donating protons and electrons (8). And tannins have been incorporated into capsule membranes to confer antioxidant and metal-reducing properties (9). The ease with

which electrons can flow through phenolics via redox reactions is important not only for technology, but also for our health (10, 11), our diseases (12, 13), and our environment (14). Potentially, self-assembly of the Fe^{III}-tannin films may do more than organize structure—it may also serve to localize activities that are capable of interfacing with our natural world.

Although the old adage from the pulp and paper industry may still ring true “that you can make anything from lignin but money,” the reported recent discoveries (1, 4) may yield important commercial opportunities for lignin’s simpler cousins. Perhaps more important, the follow-on work that these reports will invariably generate should teach us much about the structures and properties of phenolics, and these insights will help us better understand our natural world. Thus, in the case of phenolics, our technological quest may drive our understanding of nature (15).

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Acknowledgments: Supported by the Robert W. Deutsch Foundation and the U.S. Department of Defense (DTRA B0085P0008 and ONR N000141010446).

10.1126/science.1241562

GENETICS

Moving Beyond “Isolated” Gene Patents

Arti K. Rai¹ and Robert Cook-Deegan²

On 13 June 2013, the United States Supreme Court handed down its highly anticipated decision in *Association of Molecular Pathology (AMP) v. Myriad Genetics (1)*. A unanimous Court held that genes and the information they encode are not patent-eligible subject matter “simply because they have been isolated.” Hewing closely to the position of the U.S. solicitor general, who represents the executive branch of the federal government before the Supreme Court, the Court argued that DNA that has merely been isolated (genomic DNA or gDNA) is a “product of nature” and not eligible to be patented, whereas DNA with introns removed (complementary DNA or cDNA) is patent-eligible (introns are DNA sequences that do not encode a gene product). The Court also appeared to adopt the

solicitor general’s underlying economic logic that drawing the difficult line between what subject matter should and should not be patent-eligible requires respecting the “delicate balance” that patent law strikes between patent claims that create incentives for innovation and claims that block further innovation. Under this “well-established” balancing approach, gDNA claims that cover broad categories of information rather than “the specific chemical composition of a particular molecule” are suspect. Informational content is, however, only one factor in the calculus.

Although the Court indicated that cDNA claims also covered information, it held that the removal of introns makes cDNA molecules patent-eligible. Because intron removal is relatively routine, the Court’s decision could be seen as stepping back to some degree from the Court’s unanimous but highly controversial opinion last year in *Mayo v. Prometheus (2)*. In that case, the Court held that adding scientifically routine activity to a “law of nature” is insufficient for patent eligibility.

The U.S. Supreme Court decides that not all gene patents are alike—what does this mean for research, innovation, business, and patients?

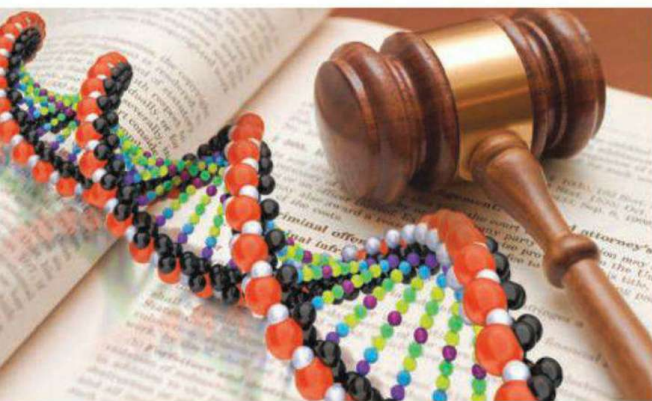
It unanimously invalidated method claims on measuring a thiopurine drug metabolite to adjust doses of a thiopurine drug, arguing that the claims in question merely added routine activity to the natural law that individuals metabolize thiopurine drugs differently.

The Court’s analysis does not connect the dots as to why claims to information in the form of cDNA are less problematic than claims to information in the form of gDNA. Nonetheless, as amicus briefs from both the solicitor general and from the renowned geneticist (and co-chair of the President’s Council of Advisors on Science and Technology) Eric Lander (3, 4) indicated, claims on cDNA can generally be worked around for research purposes. Additionally, most analysts would agree that the patents most valuable as spurs to innovation are not gDNA patents, but those on cDNA or other engineered DNA molecules (4).

The Court’s decision clearly weakens the diagnostic service monopoly model of firms such as Myriad Genetics, at least to

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the degree that this model relies on patents. Myriad Genetics offers a genetic test for *BRCA1/2* mutations. The day the opinion was announced, Ambry Genetics, GeneDx, DNATraits, Quest Diagnostics, and Pathway Genomics, as well as a number of academic institutions stated that they would begin testing for mutations in the genes *BRCA1* and *BRCA2*, which increase the risk of breast cancer (5). The Court's opinion not only emboldens competitors that rely on traditional sequencing methods, but also reduces the fear of infringement among those that rely on whole-genome sequencing. Among



the new entries are tests not just on *BRCA1/2*, but also multigene tests that will now include *BRCA1/2* (6).

Firms and university labs are beginning to compete on price. Myriad Genetics and other companies that have relied on gDNA patents for their service monopolies will likely have to compete on other grounds, such as turnaround time, quality of testing, clarity of clinical reports, sales force, and securing payment from insurers.

The quality question is particularly salient. Myriad has performed more than a million tests for *BRCA1/2* during its period of presumed exclusivity since 1998 (7). Even before litigation arose, in November 2004, Myriad had stopped contributing mutation information to public databases. As competitors get into the market place, a key question is whether Myriad and providers with similar business models will maintain their superiority in information regarding mutations. Keeping data proprietary confers an advantage when interpreting the small percentage of *BRCA* test results whose clinical importance cannot be discerned from public data sources. Patients seeking testing, however, will now have options that include laboratories contributing data to public databases. Two of the competing laboratories, GeneDx and Ambry Genetics, are launching *BRCA* testing under a “free the data” ban-

ner, as an appeal to women and physicians. In the near term, at least, the market will pit Myriad's advantage of having a huge proprietary database built on its period of monopoly against firms promoting medical progress through data access and open science.

More generally, concerns about whole-genome sequencing being impeded by gDNA patents are now gone. Dissipating the shadow of patent infringement liability that hung over whole-genome sequencing was an important factor that motivated officials in the U.S. National Institutes of Health and U.S. Office of Science and Technology Policy to persuade the solicitor general to reject the U.S. Patent and Trademark Office's position allowing claims on “isolated” DNA molecules. In the future, agencies outside the traditional patent community may continue to play a role. For example, the U.S. Department of Health and Human Services, which administers the Affordable Care Act (ACA), Medicare, and Medicaid, may deem it appropriate to invoke the ACA's mandated coverage of *BRCA1/2* testing to increase the public accessibility of mutations information. In other countries with more centralized health insurance systems, purchasers have played a much greater role in determining the terms of access to diagnostic genetic testing (8).

Even within the human diagnostic testing arena, the Court's decision arguably has only a modest practical effect. Many human gDNA patents have already expired or will do so soon. Outside this arena, it is also likely to have only a modest effect. Some analysts have expressed concern about inability to patent prokaryotic DNA, which lacks introns, or DNA products based on sequences found in nature. If DNA molecules prove useful as products, however, they will likely not be “merely isolated.” And if such products are indeed merely isolated, then the claims are likely directed broadly at research uses, the very activities the Court was deliberately protecting from patent exclusivity.

An important lingering question involves the opinion's effect on patented molecules other than DNA that have indeed “simply” been isolated from nature. In its amicus brief, the Biotechnology Industry Organization listed an array of such molecules, many of which have important therapeutic uses (9). These include rapamycin and tacrolimus, naturally produced by bacteria. In each of these cases, however, the patents claim the type of “specific chemical composi-

tions” that the Supreme Court viewed favorably in the Myriad case.

Much of the emotional force behind the challenge to the Myriad patents emerged from concerns about patient access and control of medical decision-making. Indeed, the case would probably never have arisen had other policy levers—nonexclusive licensing norms for university-generated diagnostic patents and industry traditions of refraining from suing physician-researchers—been used. Moreover, as noted, the most obvious impact of the decision may be increased access, reduced price, and perhaps most importantly, the emergence of multigene first-line genetic tests for inherited risk of breast and ovarian cancer—replacing the current multistep process of testing first for just two genes.

Nonetheless, the Court's opinion views patent eligibility through the conventional lens of innovation. Indeed, although research and patient access cannot entirely be separated in the case of physician-researchers who also provide clinical care, the opinion makes no mention of patient access, price, or control over medical practice. Whether any of the Supreme Court justices were influenced by concerns other than innovation is a question we cannot answer. Fortunately, for those preeminently concerned about innovation, the Court's decision should, if read narrowly and through the lens of economic policy, largely preserve innovation incentives.

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Acknowledgments: R.C.-D. is supported by the Ewing Marion Kaufmann Foundation. R.C.-D. and A.K.R. are supported by National Human Genome Research Institute through grant P50 HG003391. The views expressed here do not necessarily reflect those of the funders.

Published online 27 June 2013.

10.1126/science.1242217

EVOLUTION

A Muscular Perspective on Vertebrate Evolution

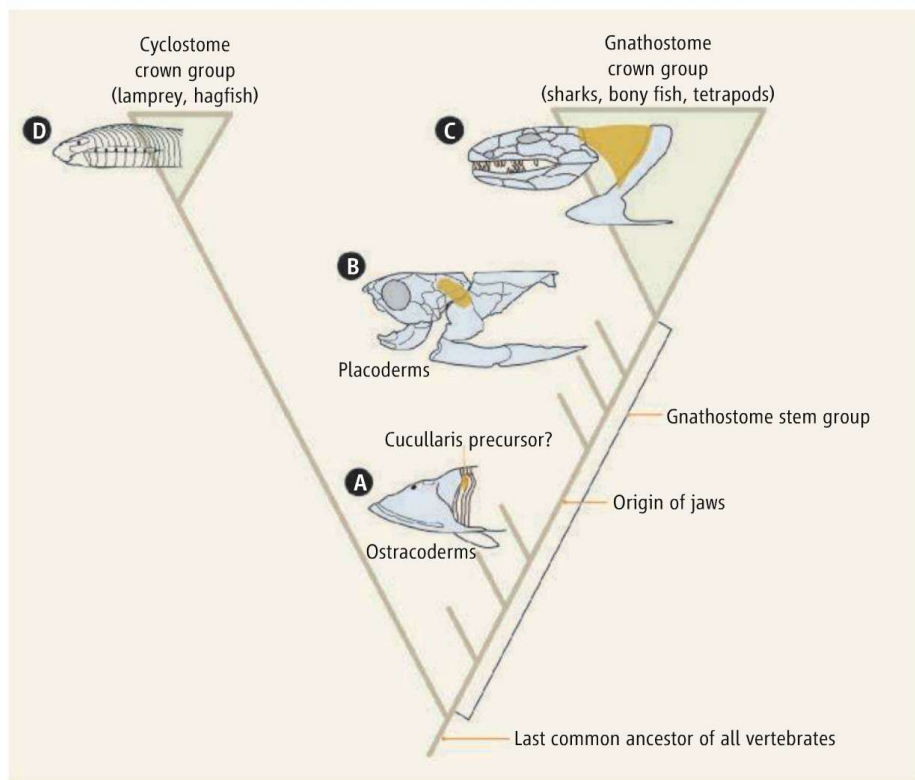
Shigeru Kuratani

Most living vertebrates, from sharks to shrews and from hummingbirds to humans, are “jawed vertebrates,” distinguished by their possession of a jaw. These animals share other traits as well, such as two sets of paired appendages, shoulder girdles, and dual nostrils, revealing the basic anatomical architecture inherited from a common ancestor. Another group of vertebrates, the cyclostomes, comprises the two modern jawless vertebrates, lampreys and hagfish. These animals lack not only a jaw but also paired fins and shoulder girdles, showing that an even more ancient ancestor of vertebrates that gave rise to both the cyclostomes and jawed vertebrates also lacked these features. On page 160 this issue, Trinajstić *et al.* (1) systematically describe the muscle anatomy of three fossil animals from the earliest jawed vertebrate group, the placoderms, which evolved soon after the acquisition of the jaw.

The term gnathostome (“jawed mouth”) refers to the lineage that includes all jawed vertebrates after they diverged from the cyclostomes (see the figure). This lineage is divided into a crown group and a stem group. All living or extinct members of the crown group have the full set of jawed vertebrate characters, although paired appendages and shoulder girdles have been lost again in a few of them (like snakes). The stem group consists only of fossil forms. It is within the stem group that the anatomical transition from jawless to jawed forms took place (see the figure). The earliest and most primitive stem gnathostomes (called ostracoderms) lacked jaws, and in fact look more like armor-plated lampreys than jawed vertebrates. But further up in the stem group, just below the bottom of the crown, we find the first vertebrates with jaws; these are the placoderms, the subject of Trinajstić *et al.*’s study (see the figure).

A key feature of jawed vertebrates is the shoulder girdle, which carries the pectoral fins (or forelimbs) and is separate from

The shoulder muscles of an early jawed vertebrate represent an intermediate evolutionary state.



How vertebrates evolved. This hypothetical scenario of neck and cucullaris muscle evolution builds on the data presented by Trinajstić *et al.* and uses a simplified vertebrate phylogeny. Placoderms belong to the gnathostome stem group. *Cephalaspis*, an ostracoderm, represents the jawless state (A). The head shield of this animal appears to contain a pectoral girdle precursor, but it remains unknown whether a cucullaris muscle precursor was present (12). A placoderm with the pectoral girdle articulated with the dermal cranium (B) represents the intermediate state of neck evolution. An ancestral tetrapod with movable neck and shoulder (C) represents the current state of evolution. Cyclostomes such as the lamprey lack a cucullaris and do not have movable neck or jaw (D).

the head. Even before the acquisition of the jaw, some advanced ostracoderms possessed pectoral fins, but these were attached directly to the head shield (2) (see A in the figure). Jaws and a separate shoulder girdle appeared around the same time in the fossil record. Knowledge of the morphology of these earliest jawed vertebrates, especially with respect to soft tissues such as muscles, is necessary for understanding how vertebrates evolved.

Placoderms are a segment of the gnathostome stem with multiple branches (3, 4) (see B in the figure). This implies that the direct line of ancestry leading to modern jawed vertebrates runs through the placoderms, rather

than branching from a common ancestor with them; put another way, our own distant ancestors were placoderms. Although placoderms resemble present-day jawed vertebrates, they also seem to have possessed some ancestral characters, such as a more primitive pattern of eye muscles, similar to those of lampreys (5).

Trinajstić *et al.* now highlight another major difference in the anatomical patterns of muscles between placoderms and modern cartilaginous fishes (sharks and their relatives), which are often regarded as the prototypical jawed vertebrates. Placoderms seem to have been the first vertebrates to develop a movable “neck” separating the head and shoulder girdle, but it is of a very peculiar

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kind: The skull hinges against the shoulder girdle, only allowing up-and-down head movement (see B in the figure).

The neck region of extant jawed vertebrates contains a distinctive set of neck muscles (6). Precursors of hypobranchial muscles (muscles of the tongue and the floor of the mouth) are present in both cyclostomes and placoderms (7, 8). Another major component of the neck muscles is the cucullaris muscle, which stretches between the skull and shoulder blade. The cucullaris is not apparent in the lamprey (9) and is thus regarded as a defining character of the gnathostome crown group.

By observing the soft tissues preserved in fossil placoderms from the Upper Devonian Gogo Formation of West Australia, Trinajstić *et al.* found two pairs of muscles between the skull and dermal shoulder girdle, which was articulated with the skull by a hinge, or neck joint (see B in the figure); each of these muscle pairs functioned to depress and elevate the head. The authors argue that these muscles enabled the movement of the head at the hinge between shoulder girdle and skull, and that the depressor is equivalent to the cucullaris in the gnathostome crown group (see A in the figure). The muscle differs in both shape and function from the cucullaris of sharks, which is not associated with a hinge joint. Trinajstić *et al.* also note differences in

the morphology of segmented trunk muscles in placoderm from those of the shark, as well as the presence of the abdominal transverse-like muscles.

From the developmental perspective, jawed vertebrate muscles are characterized by modification of some rostral trunk muscles to create the “neck.” Myoblasts of these neck muscles can migrate over long distances to reach their targets, where they differentiate into various shapes (10). This developmental program is particularly elaborated around the time of jaw acquisition, leading to the extensive incorporation of trunk muscles into the head (such as in the case of the human tongue), as well as the acquisition of a highly movable neck. The primitive shoulder girdle in placoderms, as suggested by Trinajstić *et al.*, may be an intermediate state of neck evolution that simultaneously reveals the beginnings of a jawed vertebrate novelty, the cucullaris. The presence of the transverse abdominal muscles in placoderms is another mysterious finding of Trinajstić *et al.*, because this muscle has been thought to be present only in tetrapods. Phylogenetic importance or homology aside, this muscle is potentially similar to a component of the trunk muscle in tetrapods, the abaxial muscle (11), which also develops as the result of myoblast migration and interactions between

myoblasts and the embryonic mesenchymal environment of the lateral body wall.

If the muscle patterns reported by Trinajstić *et al.* are found to reflect the general morphology of the placoderms, it would suggest that the developmental bases for the muscle anatomy of modern jawed vertebrates were present, in primitive form, around the time of the appearance of the functional jaw. This would stimulate even greater curiosity about the anatomy of more ancient stem gnathostomes such as ostracoderms, because the beginning of the jawed vertebrate body plan is likely to be buried in the anatomy of these animals.

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10.1126/science.1241451

ENGINEERING

Nanoscale Transistors—Just Around the Gate?

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Further reduction in the size of the metal-oxide semiconductor field-effect transistors (MOSFETs) used in computer chips will require more complex geometries to enhance the gate control of the current flow in the transistor channel (1). These advanced designs allow transistor scaling (maintaining performance as size decreases) and minimize the leakage of current when the device is in the off-state. The voltage of operation can be reduced without loss of performance, making the devices function with less power dissipation per operation. The optimal MOS-

FET geometry surrounds a cylindrical channel with the gate electrode (2). This “gate-all-around” (GAA) enhances electrostatic control of the entire channel surface (see the figure), and when used with superior charge transport materials, should deliver enhanced performance. Franklin *et al.* (3) now report on nanoscale complementary MOSFETs in which suspended single-walled carbon nanotubes (SWCNTs) form the channel with a GAA geometry. This work marks a milestone in moving SWCNT nanoelectronics from laboratory prototypes toward a manufacturable technology.

The electronic properties of SWCNTs depend on their diameter and chirality. These materials are synthesized as mixtures, but scalable separation methods for electronic

Advanced geometries for gate electrodes that reduce current leakage can decrease the size of high-performance transistors.

type and chirality based on ultracentrifugation (4) and column chromatography (5) provide monodisperse SWCNT samples with well-defined properties. Franklin *et al.* used substrate-driven growth of horizontally aligned SWCNTs via chemical vapor deposition (6–8) to create their devices; recently these techniques have been combined to create chirality-controlled, aligned SWCNTs (9). This “cloning” method provides a path toward high-yield, high-throughput manufacturing of SWCNT nanoelectronic devices. Electronically, the intrinsic channel region of SWCNTs consists of ballistic conductors displaying length-independent transport at distances below ~50 nm (10). The on-current of SWCNT MOSFETs with 9-nm channel lengths exceeds that of state-of-the-art Si

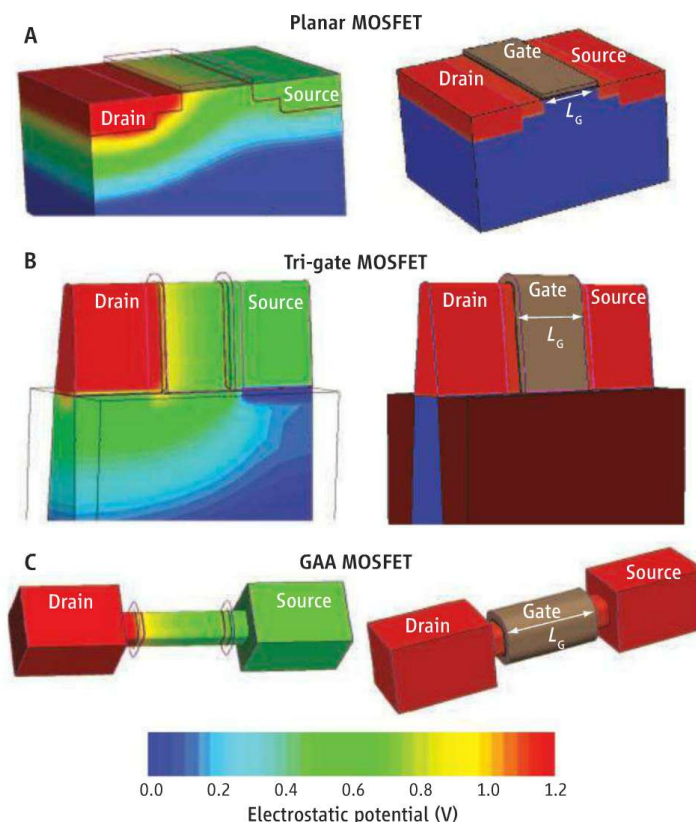
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MOSFETs with comparable dimensions (11).

Controlling the chirality, alignment, and density of SWCNT channels remains a considerable but surmountable hurdle. The lack of an established technique for controlling the spatial equilibrium carrier density (achieved in many semiconductors by substitutional doping) poses a more fundamental challenge. As intrinsic semiconductors, SWCNT devices rely on work function engineering of electrical contacts that create electron- or hole-blocking Schottky barriers (12). Franklin *et al.* achieved complementary MOSFET performance by trapping charge in the gate oxides of their devices, which electrostatically dope the channel to yield predominantly p- or n-channel devices. However, the reliability of this approach remains to be proven along with its compatibility with work function engineering of the metal source and drain contacts.

The GAA geometry is key to achieving optimal electrostatic control, but it is not specific to SWCNT channels; silicon (Si) and alternative high-mobility channel materials, including germanium and compound semiconductors ["III-V" materials such as indium-gallium-arsenide (InGaAs)], are also under investigation. Gate lengths below 15 nm in GAA transistors were recently implemented with vertical nanowires made by etching Si and with metallic source and drain contacts. These transistors displayed an immunity to drain-induced barrier lowering (DIBL; see the figure) for nanowire diameters less than 40 nm (13). However, parasitic resistance remains an issue.

Compound semiconductors offer opportunities for independent channel and source-drain engineering for transport enhancement and parasitic resistance reduction, respectively. Tomioka *et al.* (14) recently demonstrated position-controlled growth of vertically aligned InGaAs nanowires on Si by selective-area metal-organic vapor-phase epitaxy. Vertical GAA transistors were fabricated by using modulation-doped core-multishell (MD-CMS) nanowire structures in which the device layering is cylindrical rather than planar. These devices exhibit high drive current, transconductance, and excel-



lent gate electrostatics, albeit at a gate length of 200 nm. Aggressive diameter scaling via simplification of the MD-CMS multilayer structure, such as that recently proposed for a III-V trigate transistor by Radosavljevic *et al.* (15), needs to be demonstrated in GAA configuration.

Other GAA geometries are possible; Gu *et al.* (16) used top-down (lithographic) fabrication techniques to form horizontally aligned InGaAs nanowires stacked vertically, providing an additional degree of freedom to scale on-current independently from the area. This approach may circumvent packing density limitations associated with the vertical nanowire approach, yet self-heating needs to be carefully managed.

The era of nonplanar transistors is upon us as multigate (three-dimensional) transistors have begun to replace planar gate devices in high-performance computing. We expect Si compatibility to drive the choice of non-Si channel material (Ge, III-V, and SWCNTs) to be adopted first. Breakthroughs in crystal growth such as the use of stress-compliant substrates will allow selective growth of high-mobility channel materials on large-area silicon substrates. Different material solutions are likely for n-channel and p-channel devices and will necessitate heterogeneous materials integration at the wafer level. For dielec-

Optimizing transistor electrostatics. Transistors switch current on and off by modulating the height of an energy barrier that controls the flow of charge carriers through the channel between the source and the drain. Drain-induced barrier lowering (DIBL) is a detrimental short-channel effect that increases the off-state leakage current and drain-bias-dependent threshold voltage. (A) The planar n-channel MOSFET depicted here, with n-type contacts and a p-type channel region with a channel length L_G of 26 nm, displays signs of DIBL. The higher electrostatic potential of the drain (1.2 V) extends into the channel toward the source electrode. (B) The gate bias in the trigate n-channel MOSFET couples to the channel from three surfaces and reduces the impact of the drain potential on the source side of the barrier. (C) The gate-all-around (GAA) geometry further reduces the drain potential spreading, enabling nanoscale transistors with shorter L_G that do not exhibit increases in off-state leakage current.

tric barriers, low-temperature atomic-layer deposition and atomic-layer epitaxy techniques will likely be needed to produce passivation layers for nonsilicon channel materials that are prone to oxidation. The advantages of the GAA architecture make it the geometry of choice for future generations of MOSFET transistors.

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Injection-Induced Earthquakes

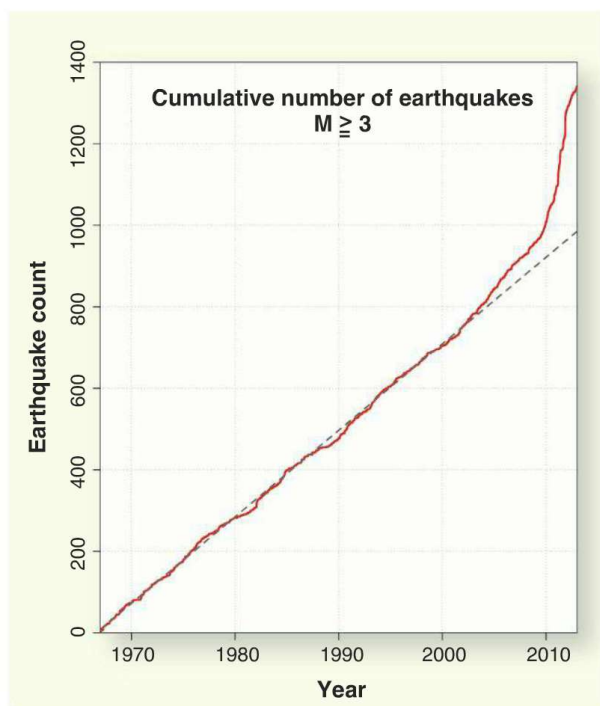
William L. Ellsworth

Background: Human-induced earthquakes have become an important topic of political and scientific discussion, owing to the concern that these events may be responsible for widespread damage and an overall increase in seismicity. It has long been known that impoundment of reservoirs, surface and underground mining, withdrawal of fluids and gas from the subsurface, and injection of fluids into underground formations are capable of inducing earthquakes. In particular, earthquakes caused by injection have become a focal point, as new drilling and well-completion technologies enable the extraction of oil and gas from previously unproductive formations.

Advances: Microearthquakes (that is, those with magnitudes below 2) are routinely produced as part of the hydraulic fracturing (or "fracking") process used to stimulate the production of oil, but the process as currently practiced appears to pose a low risk of inducing destructive earthquakes. More than 100,000 wells have been subjected to fracking in recent years, and the largest induced earthquake was magnitude 3.6, which is too small to pose a serious risk. Yet, wastewater disposal by injection into deep wells poses a higher risk, because this practice can induce larger earthquakes. For example, several of the largest earthquakes in the U.S. midcontinent in 2011 and 2012 may have been triggered by nearby disposal wells. The largest of these was a magnitude 5.6 event in central Oklahoma that destroyed 14 homes and injured two people. The mechanism responsible for inducing these events appears to be the well-understood process of weakening a preexisting fault by elevating the fluid pressure. However, only a small fraction of the more than 30,000 wastewater disposal wells appears to be problematic—typically those that dispose of very large volumes of water and/or communicate pressure perturbations directly into basement faults.

Outlook: Injection-induced earthquakes, such as those that struck in 2011, clearly contribute to the seismic hazard. Quantifying their contribution presents difficult challenges that will require new research into the physics of induced earthquakes and the potential for inducing large-magnitude events. The petroleum industry needs clear requirements for operation, regulators must have a solid scientific basis for those requirements, and the public needs assurance that the regulations are sufficient and are being followed. The current regulatory frameworks for wastewater disposal wells were designed to protect potable water sources from contamination and do not address seismic safety. One consequence is that both the quantity and timeliness of information on injection volumes and pressures reported to regulatory agencies are far from ideal for managing earthquake risk from injection activities. In addition, seismic monitoring capabilities in many of the areas in which wastewater injection activities have increased are not capable of detecting small earthquake activity that may presage larger seismic events.

Earthquakes with magnitude (M) ≥ 3 in the U.S. midcontinent, 1967–2012. After decades of a steady earthquake rate (average of 21 events/year), activity increased starting in 2001 and peaked at 188 earthquakes in 2011. Human-induced earthquakes are suspected to be partially responsible for the increase.



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<http://dx.doi.org/10.1126/science.1225942>



Cite this article as W. L. Ellsworth, *Science* **341**, 1225942 (2013). DOI: 10.1126/science.1225942

ARTICLE OUTLINE

Mechanics of Induced Earthquakes

Earthquakes Induced by Hydraulic Fracturing

Earthquakes Induced by Deep Injection

Lessons from Three Case Studies of Deep, High-Volume Injection

Other Causes of Induced Earthquakes

Hazard and Risk of Induced Earthquakes

Unknown Knowns

Reducing the Risk of Injection-Induced Earthquakes

ADDITIONAL RESOURCES

The following resources provide an introduction to earthquake hazards and risk, the science of induced earthquakes, and strategies for managing the risk.

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Injection-Induced Earthquakes

William L. Ellsworth

Earthquakes in unusual locations have become an important topic of discussion in both North America and Europe, owing to the concern that industrial activity could cause damaging earthquakes. It has long been understood that earthquakes can be induced by impoundment of reservoirs, surface and underground mining, withdrawal of fluids and gas from the subsurface, and injection of fluids into underground formations. Injection-induced earthquakes have, in particular, become a focus of discussion as the application of hydraulic fracturing to tight shale formations is enabling the production of oil and gas from previously unproductive formations. Earthquakes can be induced as part of the process to stimulate the production from tight shale formations, or by disposal of wastewater associated with stimulation and production. Here, I review recent seismic activity that may be associated with industrial activity, with a focus on the disposal of wastewater by injection in deep wells; assess the scientific understanding of induced earthquakes; and discuss the key scientific challenges to be met for assessing this hazard.

Earthquakes are expected within tectonically active regions such as along plate boundaries or within distributed zones of deformation. Recent seismic activity across the coterminous United States, for example, concen-

trates along the plate boundaries of the West Coast and within the intermountain West (Fig. 1). Within such actively deforming zones, elastic strain energy accumulates in the crust, sometimes for centuries, before being released in earthquakes. The potential for earthquakes also exists within continental interiors, despite very low deformation rates (*1*). This is because shear stress levels within the interior of plates or near plate

boundaries are commonly found to be near the strength limit of the crust (*2*). Under these conditions, small perturbations that effect fault stability can and do trigger earthquakes (*3–6*). For example, the injection of water under high pressure into impermeable basement rocks beneath Basel, Switzerland, to develop an enhanced geothermal system beneath the city induced four moment magnitude (M_w) 3 earthquakes in 2006 and 2007 (*7*) (earthquake magnitudes measured using other scales are denoted by M). These small earthquakes led to the abandonment of the project, loss of the investment, and ongoing litigation over compensation for damage. The extraction of natural gas from shallow deposits in the Netherlands also causes earthquakes (*8*). A recent M 3.4 event near Loppersum damaged scores of homes in the area, resulting in large losses for the property owners (*9*).

Within the central and eastern United States, the earthquake count has increased dramatically over the past few years (Fig. 2). More than 300 earthquakes with $M \geq 3$ occurred in the 3 years from 2010 through 2012, compared with an average rate of 21 events/year observed from 1967 to 2000. States experiencing elevated levels of seismic activity included Arkansas, Colorado, New Mexico, Ohio, Oklahoma, Texas, and Virginia. The greatest rise in activity occurred in 2011 when 188 $M \geq 3$ earthquakes occurred. Although earthquake

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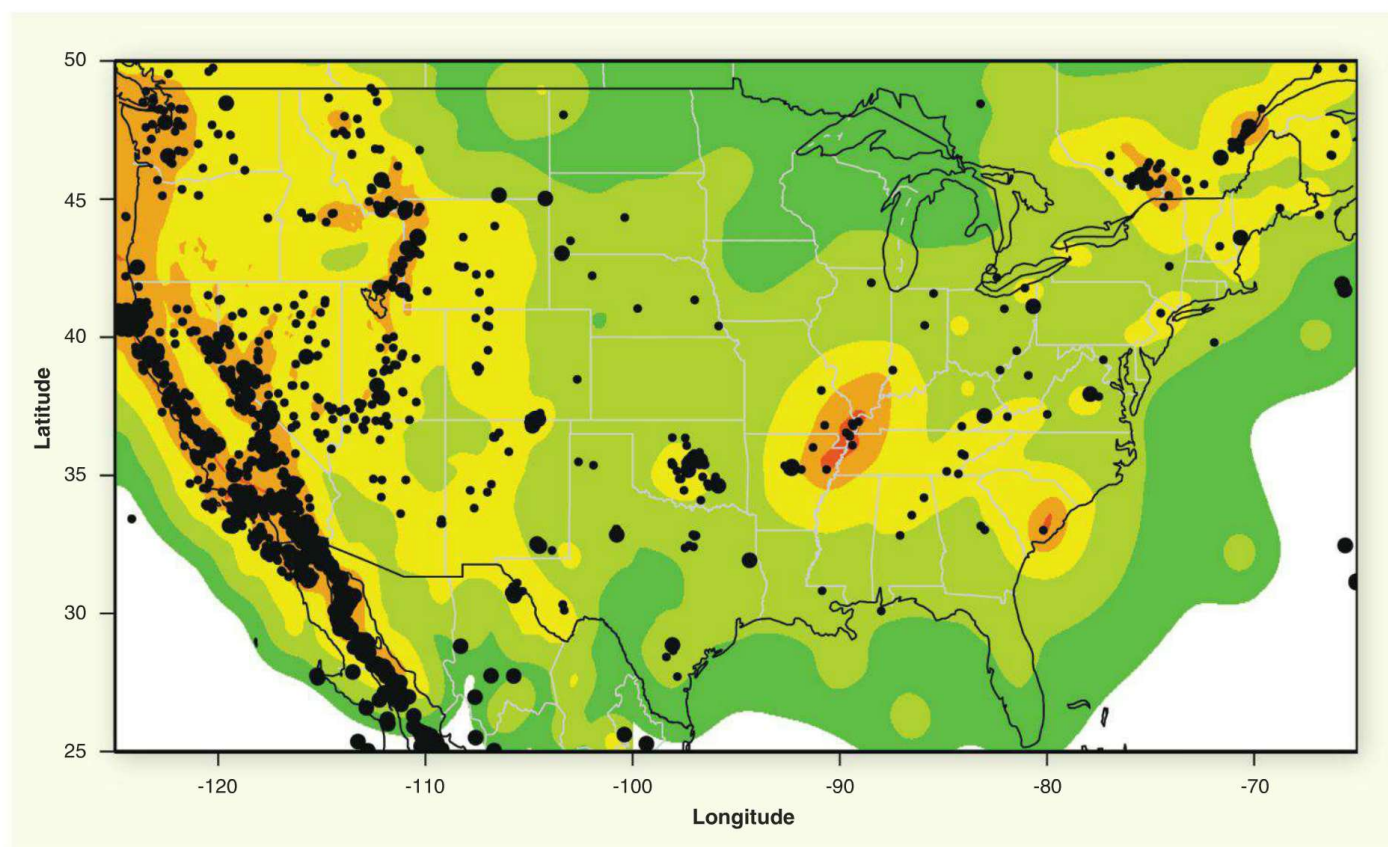


Fig. 1. Seismicity of the coterminous United States and surrounding regions, 2009–2012. Black dots denote seismic events. Only earthquakes with $M \geq 3$ are shown; larger symbols denote events with $M \geq 4$. Background colors give the

probability of peak ground acceleration with a 2% probability of exceedance in 50 years, from the U.S. National Seismic Hazard Map (*1*). Red, $\geq 1g$; orange, 0.3 to $1g$; yellow, 0.1 to $0.3g$; light green, 0.03 to $0.1g$; darker green, 0.03 to $0.1g$.

detection improved for $M < 3$ as the USArray transportable seismograph array began to pass through the region starting in 2008 (10), a recent report on seismicity in the central and eastern United States found that the probability of missing $M \geq 3$ earthquakes in the region has been near zero for decades (17). Consequently, the increased earthquake count represents a temporal change in earthquake rate. Because the hazard of damaging ground shaking is fundamentally related to the rate of earthquake occurrence (1), regions where the rate increased may be more hazardous than forecast by the 2008 version of the U.S. National Seismic Hazard Map (Fig. 1) (7). Understanding why seismicity increased and how this increase affects the hazard have become a priority for the earthquake-research community.

A number of these recent earthquakes occurred in areas where specific types of nearby industrial activities raise the possibility that these events were induced by human activity. Here, I will use the term “induced” to include both earthquakes triggered by anthropogenic causes that primarily release tectonic stress and those that primarily release stresses created by the industrial activity (4). Understanding which earthquakes may have been induced and, if so, how are challenging problems to solve in the current data-poor environment.

Several examples since 2011 highlight the difficulty in determining whether earthquakes were induced by human activity. The M_w 4.0 earthquake on 31 December 2011 in Youngstown, Ohio, appears to have been induced by injection of wastewater in a deep Underground Injection Control (UIC) class II well (12). The M_w 4.7 27 February 2011 central Arkansas earthquake has also been linked to deep injection of wastewater (13). The M_w 4.4 11 September 2011 earthquake near Snyder, Texas, occurred in an oil field where injection for secondary recovery has been inducing earthquakes for years (14). The M_w 4.8 10 October 2011 earthquake near Fanning, Texas, occurred in a region where long-term production of gas has been linked to earthquake activity (15). For others, such as the M_w 5.7 6 November 2011 central Oklahoma earthquake (16) or the M_w 4.9 17 May 2012 east Texas earthquake (17), where active wastewater-injection wells are located near their respective epicenters, the question of natural versus induced remains an active topic of research.

The potential association between deep wastewater disposal wells and earthquakes has received considerable attention due to the association of this activity with the development of tight shale formations for gas and petroleum by hydraulic fracturing, or “fracking” (5). Wells used in the U.S. petroleum industry to inject fluids are regulated as UIC class II wells. Approximately 110,000 of these wells are used for enhanced oil recovery. In addition, 30,000 class II wells in the United States are used for wastewater disposal. Of these wells, most have no detected seismicity within tens of kilometers, although a few are correlated with seismicity (18). However, this can be said with confidence only for earthquakes $M_w \geq 3$, as

smaller earthquakes are not routinely reported in the central and eastern United States. So it is possible that smaller earthquakes could be more common in the vicinity of these wells. In California, where the completeness threshold is below M_w 2, the majority of the 2300 active wastewater-injection wells are located in regions of low seismicity. As with elsewhere in the United States, a small fraction of the California wastewater wells coincide with earthquakes, which raises the question of what factors distinguish those seismically active wells from the majority of wells if the earthquakes and injection activities are related.

Mechanics of Induced Earthquakes

Earthquakes release stored elastic strain energy when a fault slips. A fault will remain locked as long as the applied shear stress is less than the strength of the contact. The failure condition to initiate rupture is usually expressed in terms of the effective stress $\tau_{\text{crit}} = \mu(\sigma_n - P) + \tau_o$, where the critical shear stress τ_{crit} equals the product of the coefficient of friction μ and the effective normal stress given by the difference between the applied normal stress σ_n and the pore pressure P (3, 19, 20). For almost all rock types, μ lies be-

tween 0.6 and 1.0, and the cohesive strength of the sliding surface, τ_o , is negligible under typical crustal conditions. Increasing the shear stress, reducing the normal stress, and/or elevating the pore pressure can bring the fault to failure, triggering the nucleation of the earthquake (Fig. 3). Once initiated, sliding resistance drops and seismic waves radiate away, driven by the imbalance between the elastic stress stored in the surrounding rock mass and the frictional resistance of the dynamically weakened sliding surface. Rupture will continue to propagate, as long as the wave-mediated stress at the rupture front exceeds the static strength, and may extend into regions where the ambient stresses are below the failure threshold.

Rocks fail in tension when the pore pressure exceeds the sum of the least principal stress, σ_3 , and the tensile strength of the rock, forming an opening-mode fracture that propagates in the plane normal to σ_3 . The industrial process of hydraulic fracturing commonly involves both tensile and shear failure. Depending on the local stress state, hydraulically conductive fractures may be induced to fail in shear before $P = \sigma_3$. A successful “frac job” may create a fracture network dominated by pathways created by shear failure (21).

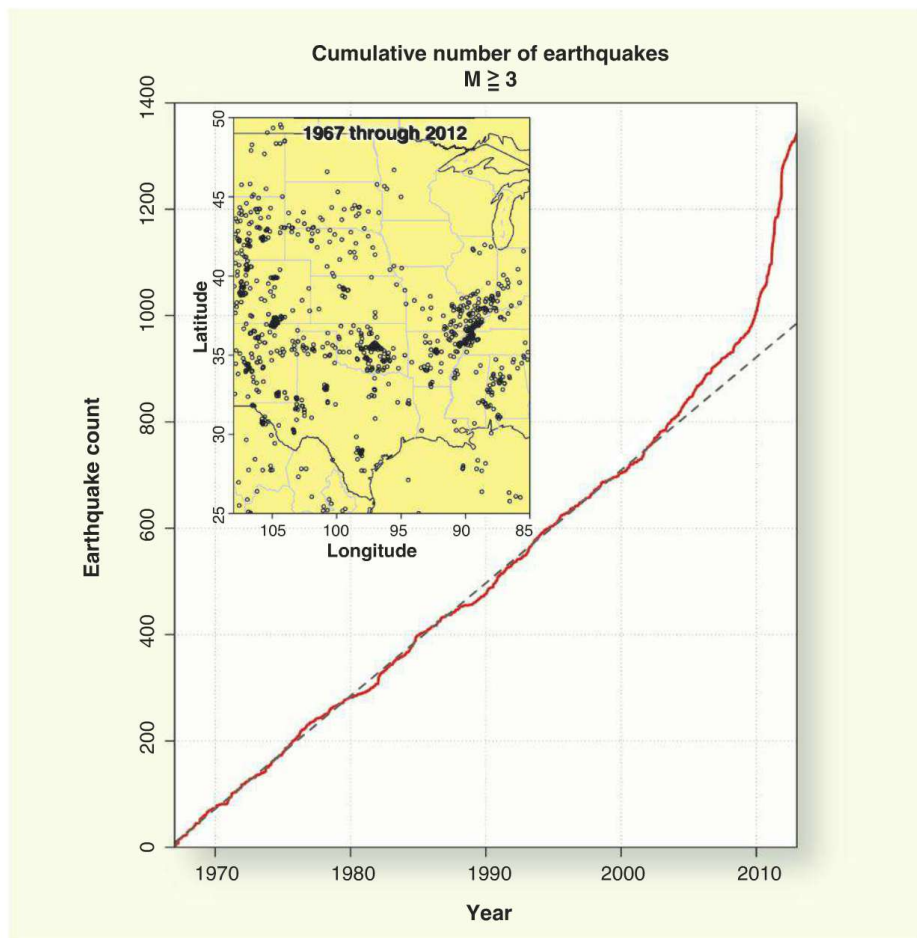


Fig. 2. Cumulative count of earthquakes with $M \geq 3$ in the central and eastern United States, 1967–2012. The dashed line corresponds to the long-term rate of 21.2 earthquakes/year. (Inset) Distribution of epicenters in the region considered here.

Earthquakes are known to be induced by a wide range of human activities (3–5) that modify the stress and/or pore pressure (Fig. 3). At present, with the use of seismological methods, it is not possible to discriminate between man-made and natural tectonic earthquakes. Induced earthquakes sometimes occur at the source of the stress or pressure perturbation; at other times, these events take place deep below and kilometers away from the source. When removed from the source, induced earthquakes typically release stored tectonic stress on preexisting faults, as do natural earthquakes. Sometimes induced events occur shortly after the industrial activity begins, but in other cases they happen long after it has been under way or even ceased. Factors that should enhance the probability of a particular stress or pore-pressure perturbation inducing earthquakes include the magnitude of the perturbation, its spatial extent, ambient stress condition close to the failure condition, and the presence of faults well oriented for failure in the tectonic stress field. Hydraulic connection between the injection zone and faults in the basement may also favor inducing earthquakes, as the tectonic shear stress increases with depth in the brittle crust (2). In addition, the larger the fault, the larger the magnitude of earthquakes it can host.

Methods for anticipating the time of failure have long been the “holy grail” of seismology (22). Though short-term prediction remains an elusive goal, it has been proposed that critically loaded faults have enhanced triggering susceptibility to dynamic stresses from distant earthquakes (23). Specifically, some but not all of the sites where fluid-injection-induced earthquakes are suspected of contributing to the recent increase in seismicity in the midcontinent (Fig. 2) experienced increased rates of microearthquakes

in the days immediately after three recent great earthquakes (23).

Earthquakes Induced by Hydraulic Fracturing

The industrial process of hydraulic fracturing involves the controlled injection of fluid under pressure to create tensile fractures, thereby increasing the permeability of rock formations. It has been used for well over half a century to stimulate the recovery of hydrocarbons. For many decades, the primary application was to improve the output of aging oil and gas reservoirs. Beginning in the late 1990s, technologies for extracting natural gas and oil from tight shale formations led to the development of new natural gas fields in many parts of the central and eastern United States, western Canada, and Europe. Global development of oil and gas from shale will undoubtedly continue, as the resource potential is high in many parts of the world.

Extracting hydrocarbons from shale requires the creation of a network of open fractures connected to the borehole. Horizontal drill holes extending up to several kilometers within the shale formation undergo a staged series of hydraulic fractures, commonly pressurizing a limited section of the cased well at a time to stimulate the flow of gas or oil into the well. Each stage involves the high-pressure injection of water into the formation. Fracking intentionally induces numerous microearthquakes, the vast majority with $M_w < 1$.

Several cases have recently been reported in which earthquakes large enough to be felt but too small to cause structural damage were associated directly with fracking. These cases are notable because of the public concern that they raised, despite maximum magnitudes far too small to cause structural damage. Investigation of a sequence of

felt events with maximum M 2.9 in south central Oklahoma revealed a clear temporal correlation between fracking operations in a nearby well and the seismic activity (24). Available data were insufficient to definitely rule out a natural cause due to the occurrence of some natural seismicity in the general area. In April and May 2012, a series of induced earthquakes with maximum M 2.3 occurred near Blackpool, United Kingdom (25), during fracking to develop a shale gas reservoir.

One of the major shale plays in the United States—the Marcellus Shale of the Appalachian Basin in Pennsylvania, West Virginia, Ohio, and New York—lies within a region characterized by low levels of natural seismic activity (Fig. 1). The regional seismographic network operated by Lamont Doherty Earth Observatory (LDEO) systematically catalogs all earthquakes with $M \geq 2$ in Pennsylvania (Fig. 4). Although thousands of hydraulic fractures were done in Pennsylvania since major development of the field began in 2005, only six earthquakes with $M \geq 2$ were detected by the LDEO network within the footprint of the Marcellus Shale, the largest of which was just M 2.3. The largest earthquake in the region since the development of shale gas happened across the Ohio border in Youngstown, where it was induced by injection (12), much of the fluid apparently coming from wells in Pennsylvania.

Beginning in 2009, an unusual sequence of earthquakes was noted in the Horn River Basin of British Columbia, including 21 events with M_w 3.0 and larger. Only the largest, at M_w 3.6, was reported as felt by workers in this remote area where it did no damage (26). The investigation into the cause of these events by the BC Oil and Gas Commission (26) concluded that the events “were caused by fluid injection during hydraulic fracturing in proximity of pre-existing faults.” Two of the hydrofrac treatments were recorded by dense seismometer deployments at the surface. Precise hypocentral locations showed that the induced earthquakes occurred on previously unknown faults located outside of the stimulation interval that were well oriented for failure in the ambient stress field. Apparently, fracture pressure was quickly communicated through hydraulically conductive pathways and induced slip on critically stressed faults via reduction of the effective normal stress.

Earthquakes Induced by Deep Injection

There has been a growing realization that the principal seismic hazard from injection-induced earthquakes comes from those associated with disposal of wastewater into deep strata or basement formations (5). Before 2011, the M_w 4.8 event on 9 August 1967 near Denver, Colorado, was the largest event widely accepted in the scientific community as having been induced by wastewater injection (5). The hazard landscape of what is possible has shifted due to the role that wastewater injection into a depleted oil field may have played in the M_w 5.7 6 November 2011 central Oklahoma earthquake (16), although a consensus on its origin has not yet been reached (27). This earthquake damaged homes and unreinforced masonry buildings in the epicentral

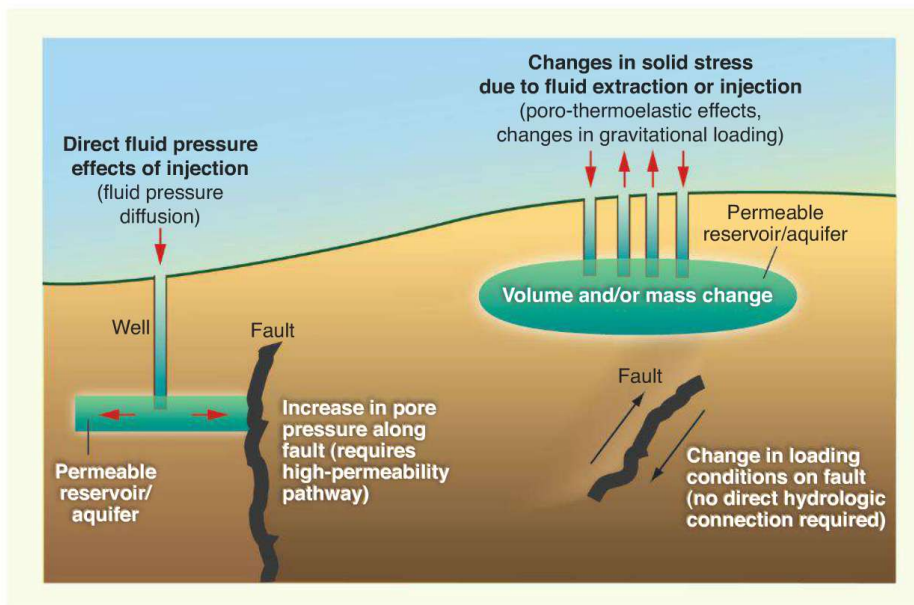


Fig. 3. Schematic diagram of mechanisms for inducing earthquakes. Earthquakes may be induced by increasing the pore pressure acting on a fault (left) or by changing the shear and normal stress acting on the fault (right). See (4).

area and was felt as far as 1000 km away in Chicago, Illinois.

The November 2011 central Oklahoma earthquake sequence initiated very close to a pair of wastewater-injection wells where disposal operation began 18 years earlier (16). No unusual seismicity was detected in this historically quiet region, where only a few events of $M < 2$ were noted, until a M_w 4.1 earthquake occurred near the wells in early 2010. Aftershocks of this event continued sporadically through 2010 and into mid-2011. This decaying sequence was shattered by a M_w 5.0 earthquake on 5 November 2011, followed 20 hours later by the M_w 5.7 mainshock. With the initiating point of the November sequence within 1.5 km of the injection wells and some earthquake hypocenters at the same depth as injection, the potential for a causal connection between injection and the earthquakes is clear. The long delay between the start of injection and the earthquakes, however, deviates from the pattern seen in other documented cases of injection-induced seismicity, such as the 2011 Youngstown, Ohio, earthquake where there was, at most, a few months of delay before induced seismicity began. In the Oklahoma case, years of injection may have been needed to raise the pore pressure above the preproduction level in this depleted oil field before fault strength was exceeded (16).

Much of the concern about earthquakes and fracking centers on the injection of wastewater, composed of flowback fluids and coproduced formation brine in deep wells, and not on fracking itself. Wastewater disposal appears to have induced both the 2011 central Arkansas earthquake (13) and the 2011 Youngstown, Ohio, earthquake (12), as mentioned above. Unprecedented levels of seismicity have also been seen in the Barnett Shale in north central Texas, where commercial development of shale gas was pioneered. Since development began in late 1998, nine earthquakes of $M \geq 3$ occurred, compared with none in the preceding 25 years. A notable sequence occurred in the Dallas–Fort Worth area from October 2008 through May 2009. A detailed investigation of this sequence concluded that the earthquakes were most probably caused by disposal of shale gas wastewater in a UIC class II disposal well at the Dallas/Fort Worth International Airport (28), although as with the Oklahoma earthquake, not all investigators agree that the case is proven (29). Because routine earthquake reporting in the region is incomplete for events of $M < 3$, the passage of the USArray Transportable Array through the region over an 18 month period in 2009–2011 made it possible to improve magnitude completeness to M 1.5 and location accuracy by several fold. Epicenters for the most reliable locations were clustered in eight groups, all within 3 km of high-rate ($>25,000$ m³/month) wastewater-injection wells (18). These results suggest that the injection rate, as well as the total

volume of injection, may be a predictor of seismic potential.

Lessons from Three Case Studies of Deep, High-Volume Injection

Conclusions about the cause of many of the recent earthquakes suspected of being induced by injection are complicated by incomplete information on the hydrogeology, the initial state of stress and pore pressure, the pumping history of the well(s), and where pressure changes are being communicated at depth. Routine earthquake locations with uncertainties of 5 to 10 km and a high magnitude-detection threshold are of limited use. Three particularly well-documented cases of injection-induced seismicity from Colorado illustrate what can be learned when more is known about the pre-injection stress state and seismicity, as well as the injection history.

Rocky Mountain Arsenal

In 1961, a deep injection well was drilled at the Rocky Mountain Arsenal (RMA) northeast of Denver, Colorado, to dispose of hazardous chemicals produced at this defense plant (30). Within several months of the start of routine injection in the 3.6-km-deep well in March 1962, residents of the northeastern Denver area began to report earthquakes, and events registered on two nearby seismic stations. Between the start of injection and its termination in February 1966, a total of 13 earthquakes with body wave magnitudes (m_b) 4 and larger occurred. The following year, the three largest of the Denver earthquakes occurred, including the M_w 4.8 event on 9 August 1967 that caused minor structural damage near the epicenter. By this time, the earthquakes had migrated as far as 10 km from the injection point (31). Hydrologic modeling showed that the migrating seismicity would track a critical pressure front of 3.2 MPa (32). Although declining,

earthquake activity continued for the next two decades, including a m_b 4.3 earthquake on 2 April 1981. The RMA earthquakes demonstrate how the diffusion of pore pressure within an ancient fault system can initiate earthquakes many kilometers from the injection point, delayed by months or even years after injection ceased.

Rangely

The insights gained from RMA led to the suggestion that earthquakes could be controlled by modulating the fluid pressure in the fault, according to the effective-stress relation (19). In 1969, the U.S. Geological Survey (USGS) began an experiment to test the effective-stress hypothesis in the Rangely oil field in northwestern Colorado (20). Water injected into the reservoir under high pressure had been used to enhance oil production at Rangely since 1957. The operator, Chevron Oil Company, gave USGS permission to regulate the fluid pressure in a portion of the field that was known to be seismically active. Laboratory measurements of the coefficient of friction on core samples of the reservoir rocks and in situ determination of the state of stress led to the prediction that a critical fluid pressure of 25.7 MPa would be required to induce earthquakes. Two cycles of fluid injection and withdrawal were conducted between 1969 and 1973. When the pressure in a monitoring well exceeded the target pressure, earthquake activity increased; when pressure was below the threshold, earthquake activity decreased. In particular, the earthquake activity ceased within 1 day of the start of backflow in May 1973, providing strong evidence that the rate of seismicity could be controlled by adjusting the pore pressure at the depth where earthquakes initiate, if stress conditions and the strength of the faulted rock mass were known. The rapid response of seismicity at the onset of

backflow also emphasized the importance of understanding the geohydrology and, in particular, the importance of hydraulically conductive faults and fractures for transmitting pore pressure within the system.

Paradox Valley

An ongoing fluid-injection project has been under way since 1996 in Paradox Valley in southwestern Colorado, where the saline shallow water table is being suppressed by pumping to prevent salt from entering the Dolores River as it crossed the valley and, eventually, the Colorado River further downstream (33). In its natural state, the Dolores River picks up salt from the groundwater as it crosses Paradox Valley. After extensive study of alternatives, the U.S. Bureau of Reclamation determined that high-pressure injection of brine into a deep disposal well (UIC class V) provided the best method for reducing the salinity of the Dolores River. Injection occurs in a tight, but highly fractured dolomitic limestone with a fracture-dominated porosity of less than 6% located 4.3 km

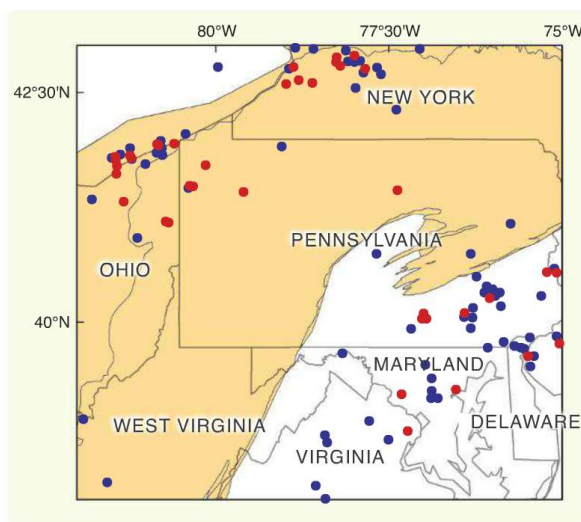


Fig. 4. Seismicity of Pennsylvania and surrounding regions, 1970–2012. Shading indicates areas underlain by deposits of the Marcellus Shale. Blue dots, earthquakes before 2005; red dots, after 2005. Seismicity was determined by the Lamont Doherty Earth Observatory (45).

below land surface. To date, more than 7×10^6 m³ of brine have been injected. One operational objective, based on both the RMA and Rangely experiences, was the need to minimize the magnitude of earthquakes induced by injection.

A local seismic network was established in 1985 to determine background levels of seismicity before the drilling of the well and initial injection tests. Between 1985 and June 1996, only three tectonic earthquakes were detected within 15 km of the well and just 12 within 35 km (33). However, hundreds of earthquakes were induced during injection tests conducted between 1991 and 1995. Most of these earthquakes were concentrated within 1 km of the injection point, although a few were located 3 to 4 km from this site. All events were below M 3. The occurrence

of induced earthquakes is not notable here, as injection required a bottom hole pressure in excess of the hydraulic fracture pressure of 70 MPa.

High injection pressure was needed to keep pace with the disposal requirements; consequently, induced earthquakes were expected when disposal operations went into production in 1996. Continuous monitoring of injection pressures and volumes, along with seismicity, is being conducted to insure the safe operation of the project. During the first few years of operations, several of the induced earthquakes exceeded M 3, necessitating changes in injection procedures in an attempt to limit the maximum magnitude. The dimension of the activated zone also grew, with earthquakes as far as 8 km from the injection point appearing within a year and events to beyond 12 km several

years later (Fig. 5). Because seismicity rapidly abated after each injection test, it was hypothesized that occasional shutdowns of 20 days would allow the fluid pressure to equilibrate, reducing the potential for larger events (33). By itself, this procedure proved inadequate, as a M 4.3 event was induced in May 2000.

After this earthquake, a new procedure was introduced in 2000 that involved periodic 20-day shutdowns and a 33% reduction in the injection volume, which initially reduced the required bottom hole pressure to 78 MPa. Over the following decade, the pressure required to inject that volume steadily increased to more than 84 MPa in 2012, drawing the revised strategy into question, as a steadily increasing injection pressure is not sustainable in the long term. On 24 January

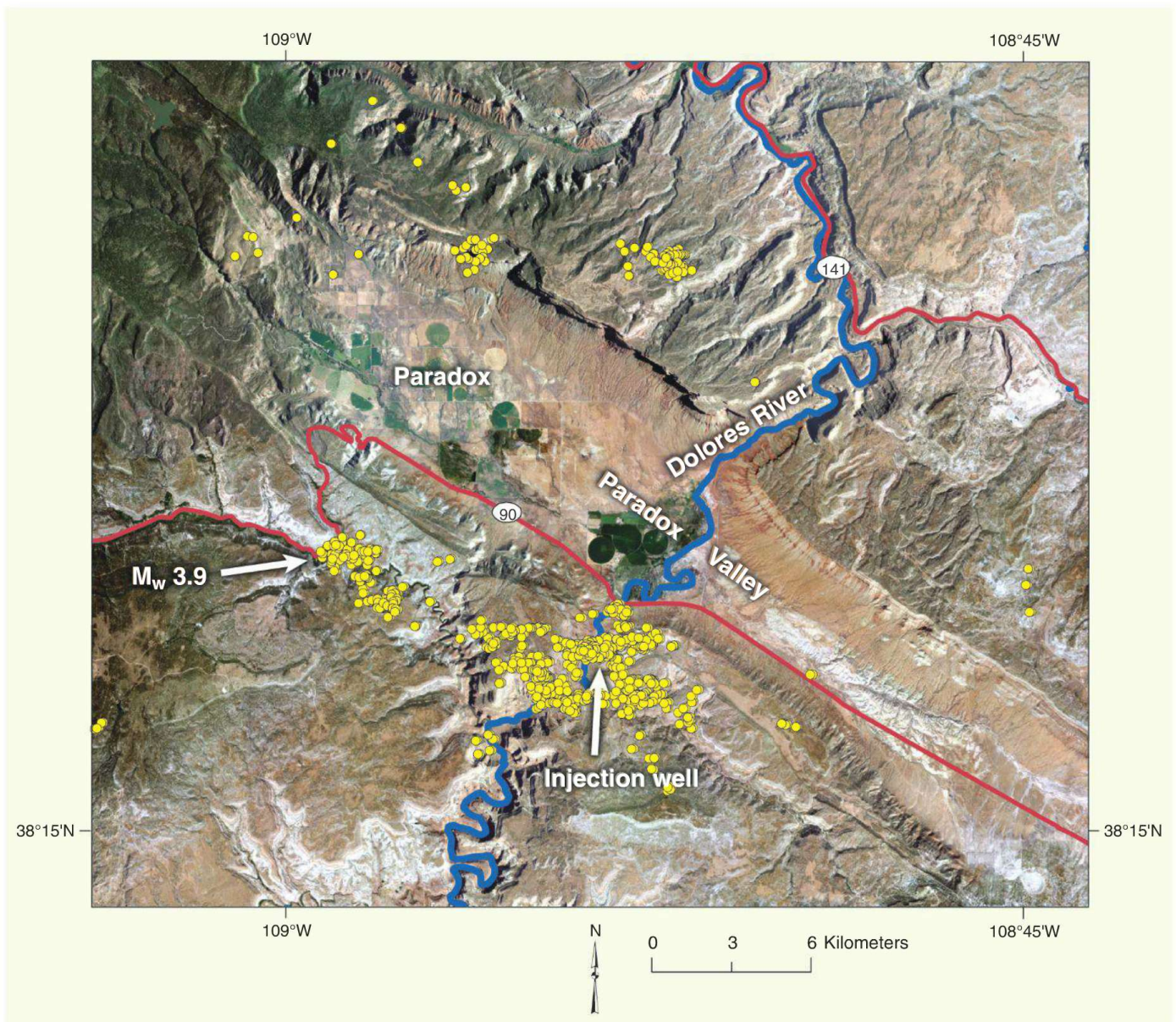


Fig. 5. Seismicity near Paradox Valley, Colorado. The U.S. Bureau of Reclamation extracts saline groundwater from shallow wells where the Dolores River crosses Paradox Valley to prevent its entry into the Colorado River system. Since 1996, the

brine has been disposed of by injection into a 4.3-km-deep UIC class V well. Injection has induced more than 1500 earthquakes with $M \geq 1$, including the M_w 3.9 earthquake on 25 January 2013, which was located 8 km northwest of the well.

2013, a M_w 3.9 earthquake occurred 8 km northwest of the well in a previously active cluster, causing strong shaking in the town of Paradox, Colorado (Fig. 5). As a consequence, injection was halted for 12 weeks before restarting at a reduced rate. The Paradox Valley experience illustrates how long-term, high-volume injection can lead to the continued expansion of the seismically activated region and the triggering of large-magnitude events many kilometers from the injection well more than 15 years after observation of the initial seismic response. This case study also illustrates the challenges for managing the risk once seismicity has been induced.

Other Causes of Induced Earthquakes

According to the effective-stress model described above, earthquakes can be induced by either reducing the effective normal stress or raising the shear stress (3–5). It has been known for decades that large reservoirs can induce earthquakes either from the effect of the elastic load of the reservoir or by diffusion of elevated pore pressure (34). Well-known examples include the deadly 1967 M 6.3 earthquake in Koyna, India (35). Yet, establishing a causal connection can be difficult when natural seismicity occurs nearby. For example, the debate about the role of the Zipingpu reservoir in triggering the M_w 7.9 2005 Wenchuan, China, earthquake may never be resolved (36, 37). What is clear, however, is that deep reservoirs in tectonically active zones carry a real risk of inducing damaging earthquakes.

Earthquakes throughout the world are also recognized to be associated with mining, petroleum and gas production, and geothermal energy extraction. Withdrawal of large volumes of fluid or gas from a reservoir or creation of a void space in a mine may modify the state of stress sufficiently to induce earthquakes that relax the stress perturbations (4). Production may also release tectonic stress. The long-term pumping of groundwater may have induced the deadly M_w 5.1 earthquake in Lorca, Spain, on 11 May 2011 (38). Pore-pressure changes alone can also induce seismicity, such as by waterflooding for secondary recovery of oil or to maintain the fluid level in a geothermal reservoir, or when a mine is abandoned and allowed to flood (3, 4). The physical connection between operational parameters such as injected volume and the seismic response can be complex. In the Salton Sea Geothermal Field, for example, the seismicity rate positively correlates with the net volume of produced fluid (extraction minus injection) rather than net injection, as would be expected if seismicity rate simply tracked pore pressure (39). This underscores the importance of geomechanical modeling for transferring understandings developed in one setting to others.

Hazard and Risk of Induced Earthquakes

The hazard from earthquakes depends on proximity to potential earthquake sources, their magnitudes, and rates of occurrence and is usually expressed in probabilistic terms (1, 40). The U.S. National

Seismic Hazard Map, for example, gives the exceedance probabilities for a variety of ground-motion measures from which the seismic design provisions in the building codes are derived (Fig. 1) (1). Our understanding of the hazard will evolve as new information becomes available about the underlying earthquake sources, which are ideally derived from a combination of fault-based information and historical seismicity. Accounting for the hazard of induced earthquakes, however, presents some formidable challenges.

In the current U.S. map (Fig. 1), for example, the estimated hazard in most parts of the central and eastern regions of the country derives exclusively from historical seismicity. How should increases in the earthquake rate since 2009 (Fig. 2) be incorporated in the model? Should identified or suspected induced earthquakes be treated the same as or differently than natural events? In particular, do induced earthquakes follow the same magnitude-frequency distribution models as natural earthquakes? This issue has particular importance, as the high end of the magnitude distribution, where events are infrequent, contributes disproportionately to both the hazard and risk. Although injection-induced earthquakes have done only minor damage in the United States to date (5), the 2011 central Oklahoma earthquake was the same magnitude as the 1986 San Salvador, El Salvador, tectonic earthquake that killed more than 1500 people, injured more than 10,000, and left 100,000 homeless (41). Losses on this scale are unlikely in North America and northern Europe, where a catastrophic building collapse in a M_w 5.7 earthquake is unlikely, but the same cannot be said for large portions of the world where nonductile concrete frame or unreinforced masonry buildings are prevalent. The earthquake that killed nine and caused serious damage Lorca, Spain, was even smaller at M_w 5.1 (40). The heavy losses in this possibly induced earthquake resulted from the exposure of many fragile buildings to strong shaking from this very shallow-focus earthquake (42). This event should serve as a reminder that risk is the product of the hazard, exposure, and vulnerability.

Unknown Knowns

Ignorance of the things that we understand we should know but do not leaves us vulnerable to unintended consequences of our actions. The effective-stress model provides straightforward guidance for avoiding induced earthquakes but requires knowledge that we rarely possess of the stress state and pore pressure acting on the fault. Quantitative predictions from the model depend on knowing initial stress and pore-pressure conditions and how perturbations to those conditions due to injection will affect the surroundings. For example, pore-pressure changes in a fault kilometers from the injection point depend on the hydrologic characteristics of connecting pathways that will, in all likelihood, be poorly known. The seismic response might not take place immediately, and decades may elapse before a damaging event occurs, as illustrated by the recent Paradox Valley

earthquake and possibly the central Oklahoma earthquake as well. Simply injecting water by gravity feed (pouring it down the well with no surface pressure) sounds safe enough. But if the deep aquifer system was originally underpressured and the faults were in frictional equilibrium with the stress (2), this apparently benign type of injection can bring faults to failure by raising the water table and, hence, the pore pressure acting on the faults.

The fact that the great majority of UIC class II injection wells in the United States appear to be aseismic, at least for earthquakes $M_w > 3$, suggests that ambient conditions in geologic formations commonly approved for disposal are far enough removed from failure that injection can be done with low risk, provided that the pressure perturbation remains confined within the intended formation. The largest injection-induced events have all involved faulting that is considerably deeper than the injection interval (13, 16, 30, 43), suggesting that transmission of increased pressure into the basement elevates the potential for inducing earthquakes. Consequently, detection of seismicity in the vicinity of the well or changes in seismicity in the neighborhood should prompt reevaluation of the hazard.

License and operational requirements for UIC class II wells in the United States are regulated under the Safe Drinking Water Act, by the U.S. Environmental Protection Agency or by delegation of authority to state agencies. The law's provisions are primarily directed toward protection of potable aquifers by requiring injection into formations deep below and geologically isolated from drinking water sources. As such, the law focuses on well integrity, protection of impermeable barriers above the injection zone, and setting operational injection pressure limits to avoid hydraulically fracturing the well. Diffusion of pore pressure into basement faults or injection pressure that would raise critically stressed faults to failure is not considered in U.S. federal regulations. From a scientific standpoint, measuring the initial stress state and pore pressure, tracking of injection history, and careful seismic monitoring would be of great value. At present, little more is required by regulation than an estimate of the fracture pressure (not to be exceeded) and monthly reporting of total injection volume and average injection pressure. In most cases, this information is not sufficient to apply the effective-stress model or gain an understanding of the hazard posed by injection activity.

Reducing the Risk of Injection-Induced Earthquakes

How can the risk of inducing damaging earthquakes through human activity be minimized in an information-poor environment? Long-term and high-volume injection in deep wells clearly carries some risk (18), even though most wells are apparently aseismic (5). In contrast, earthquakes induced during hydraulic fracturing have lower risk because of their much smaller magnitudes. The largest fracking-induced earthquakes

(24, 26) have all been below the damage threshold for modern building codes.

One approach for managing the risk of injection-induced earthquakes involves setting seismic activity thresholds that prompt a reduction in injection rate or pressure or, if seismic activity increases, further suspension of injection (44). Such “traffic-light” systems have been used selectively, going back to at least the RMA well pump tests in 1966–1967. The traffic-light system used in Basel, Switzerland (7), did not stop the four M_w 3 earthquakes from happening but might have prevented larger events. The decision to stop injection in the Youngstown, Ohio, well, based on the seismicity (12) and made the day before the M_w 4.0 event, resulted in seismicity near the well declining within a month. All of these examples feature better seismic monitoring capabilities than currently exist in much of the United States or most of the rest of the world. Lowering the magnitude-detection threshold in regions where injection wells are concentrated to below M_w 2 would certainly help, as a traffic-light system using the current U.S. detection threshold of M_w 3 in many of these areas would have limited value. Improvements in the collection and timeliness of reporting of injection data to regulatory agencies would provide much-needed information on hydrologic conditions potentially associated with induced seismicity. In particular, daily reporting of volumes, peak, and mean injection pressures would be a step in the right direction, as would measurement of the pre-injection formation pressure.

Ultimately, better knowledge of the stress and pressure conditions at depth; the hydrogeologic framework, including the presence and geometry of faults; and the location and mechanisms of natural seismicity at a few sites will be needed to develop a predictive understanding of the hazard posed by induced earthquakes. Industry, regulatory agencies, and the public are all aware that earthquakes can be induced by fluid injection. Industry needs clear requirements under which to operate, regulators must have a firm scientific foundation for those requirements, and the public needs assurance that the regulations are adequate and are being observed.

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Acknowledgments: This paper is a contribution of the USGS’s John Wesley Powell Center Working Group on Understanding Fluid Injection Induced Seismicity. I thank L. Block, C. Frohlich, S. Hickman, W. Leith, A. McGarr, J. Rubinstein, and an anonymous reviewer for insightful comments during the development of this paper.

10.1126/science.1225942

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Autonomic Nerve Development Contributes to Prostate Cancer Progression

Claire Magnon,* Simon J. Hall, Juan Lin, Xiaonan Xue, Leah Gerber, Stephen J. Freedland, Paul S. Frenette*

Introduction: Cancer cells usurp the healthy tissue microenvironment to promote their survival, proliferation, and dissemination. The role of angiogenesis, the formation of new blood vessels, in solid tumor growth is well established. Whether neurogenesis, the formation of new nerve fibers, likewise contributes to tumor development and progression remains unclear. Here, studying mouse models and human tumor samples, we examined the role of the autonomic nervous system in prostate cancer growth and dissemination.

Methods: To track tumor growth and dissemination, we studied (i) mice bearing PC-3 prostate tumor xenografts that expressed luciferase and (ii) transgenic mice expressing the c-Myc oncogene under the control of the probasin promoter (Hi-Myc mice), which develop prostatic intraepithelial neoplasia that progresses to invasive adenocarcinoma. Tumors were monitored by bioluminescence, positron emission tomography (PET), and histological analyses. Sympathetic (adrenergic) and parasympathetic (cholinergic) nerve functions were assessed using chemical or surgical neural ablation, pharmacological agonists or antagonists, and genetically engineered mice. We also determined the adrenergic and cholinergic nerve densities in radical prostatectomy tissues from a cohort of 43 patients with prostate cancer.

Results: Quantitative bioluminescence and immunofluorescence analyses, combined with histological examinations, revealed that sympathetic adrenergic nerve outgrowth was critical in the early phases of cancer development. Prostate tumor xenografts developed poorly in mice that had been pretreated by chemical or surgical sympathectomy of the prostate gland, or when stromal β_2 - and β_3 -adrenergic receptors were genetically deleted. Prostate tumors were also infiltrated by parasympathetic cholinergic fibers that promoted cancer dissemination. Cholinergic-induced tumor invasion and metastasis in mice were inhibited by pharmacological blockade or genetic disruption of the stromal type 1 muscarinic receptor. Quantitative confocal microscopy analysis of radical prostatectomy specimens from patients with low-risk ($n = 30$) or high-risk ($n = 13$) prostate adenocarcinoma revealed higher overall nerve densities in high-risk tumors relative to low-risk tumors. Adrenergic fibers were increased in normal prostate tissues surrounding the human tumors, whereas cholinergic fibers infiltrated the tumor tissue. Higher densities of adrenergic and cholinergic nerve fibers were associated with poor clinical outcome, including higher preoperative levels of prostate-specific antigen (PSA), extension beyond the prostatic capsule, and biochemical recurrence.

Discussion: These results suggest that the formation of new nerve fibers within and around prostate tumors can alter tumor behavior. The autonomic nervous system appears to exert dual functions in prostate cancer: Sympathetic nerves promote early stages of tumorigenesis, whereas parasympathetic nerve fibers promote cancer dissemination. Conceivably, drugs targeting both branches of the autonomic nervous system could provide therapeutic benefit.



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Cite this article as C. Magnon *et al.*, *Science* 341, 1236361 (2013). DOI: 10.1126/science.1236361

FIGURES IN THE FULL ARTICLE

Fig. 1. Sympathetic nervous system (SNS) controls tumor engraftment in mice.

Fig. 2. SNS nerve ablation inhibits prostate tumorigenesis in Hi-Myc transgenic mice.

Fig. 3. Parasympathetic nervous system (PNS) controls prostate tumor invasion in mice.

Fig. 4. Chrm1 promotes prostate cancer progression in Hi-Myc transgenic mice.

Fig. 5. Cholinergic signal contributes to prostate cancer metastasis in xenografted mice through the type 1 muscarinic receptor Chrm1.

Fig. 6. Chrm1 controls prostate cancer metastasis in Hi-Myc transgenic mice.

Fig. 7. Human high-risk prostate adenocarcinomas are rich in adrenergic and cholinergic nerve fibers.

Fig. 8. Density of nerve fibers in human prostate cancer specimens correlates with tumor aggressiveness.

SUPPLEMENTARY MATERIALS

Figs. S1 to S12

Tables S1 to S5

The parasympathetic nervous system promotes prostate cancer dissemination in mice. Image shows bone metastases (arrowheads) detected by Na¹⁸F-PET scanning of Hi-Myc mice, a model of prostate cancer. Such metastases are not detected when the Hi-Myc mice are genetically deficient in muscarinic cholinergic receptor type 1, an essential signaling component of the parasympathetic branch of the autonomic nervous system.

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Autonomic Nerve Development Contributes to Prostate Cancer Progression

Claire Magnon,^{1,2,3*} Simon J. Hall,⁴ Juan Lin,⁵ Xiaonan Xue,⁵ Leah Gerber,^{6,7} Stephen J. Freedland,^{6,7,8} Paul S. Frenette^{1,2,3*}

Nerves are a common feature of the microenvironment, but their role in tumor growth and progression remains unclear. We found that the formation of autonomic nerve fibers in the prostate gland regulates prostate cancer development and dissemination in mouse models. The early phases of tumor development were prevented by chemical or surgical sympathectomy and by genetic deletion of stromal β_2 - and β_3 -adrenergic receptors. Tumors were also infiltrated by parasympathetic cholinergic fibers that promoted cancer dissemination. Cholinergic-induced tumor invasion and metastasis were inhibited by pharmacological blockade or genetic disruption of the stromal type 1 muscarinic receptor, leading to improved survival of the mice. A retrospective blinded analysis of prostate adenocarcinoma specimens from 43 patients revealed that the densities of sympathetic and parasympathetic nerve fibers in tumor and surrounding normal tissue, respectively, were associated with poor clinical outcomes. These findings may lead to novel therapeutic approaches for prostate cancer.

Several lines of evidence have linked the nervous system to tumor growth and progression. Migration of tumor cells along nerves—a process termed perineural invasion—correlates with poor prognosis in certain epithelial cancers, including prostate cancer (1–3). In vitro coculture experiments have shown that sensory neurons from the dorsal root ganglion promote prostate cancer cell proliferation (4). Work with in vivo model systems suggests that neural stimulation may increase tumor incidence and the number of metastases (5–8). In addition, recent retrospective clinical data suggest that breast, melanoma, or prostate cancer patients taking β -blockers have lower recurrence rates and mortality (9–13).

Whether nerve fibers infiltrate tumors and alter their behavior has not been examined in detail. The prostate stroma is abundantly innervated by the sympathetic and parasympathetic branches of the autonomic nervous system, controlling growth and maintenance of the prostate gland (14, 15) (fig. S1). Here, we tested the hypothesis that along with neoangiogenesis (16), prostate tumors are infiltrated by autonomic nerves that affect cancer development and dissemination.

Results

Altered Prostate Cancer Development After Sympathetic Nerve Ablation

To develop a mouse model of prostate cancer that would allow longitudinal monitoring of tumor growth and metastasis by bioluminescence—a non-invasive imaging method correlating with tumor volume in vivo (17)—we injected PC-3 human prostate cells stably expressing luciferase (PC-3luc) into the ventral prostate of immunodeficient nude Balb/c (*nu/nu*) mice (Fig. 1, A and B). After 11 weeks of tumor development, a time sufficient to allow nerve development and tumor cell–prostate microenvironment interactions, we examined sections of intracapsular tumor and surrounding healthy prostate tissues by histology. This analysis revealed tumor-infiltrating sympathetic fibers [adrenergic, identified by tyrosine hydroxylase (TH) staining] arising from normal prostate tissue, as well as intra-tumor parasympathetic fibers [cholinergic, identified by vesicular acetylcholine transporter (VAcHT) staining; Fig. 1C and fig. S2]. We confirmed the neural specificity of TH and VAcHT immunofluorescence analyses by coexpression of the neuron-specific cytoskeletal subunits of neurofilament-L (NF-L) or neurofilament-H (NF-H) (Fig. 1D), which mark newly formed and mature nerve fibers, respectively, in the fetal rat (18). Tumor tissues contained significantly more NF-L than NF-H staining (fig. S3), and many fibers expressed both neural markers with sprouting NF-L⁺ neurites (Fig. 1D and fig. S3). These results suggest that the tumor recruits newly formed nerves in the stroma.

To assess the functional role of the sympathetic nervous system (SNS), we ablated adrenergic nerves by injecting 6-hydroxydopamine (6OHDA) into the tumor-bearing mice. 6OHDA treatment specifically destroyed TH⁺ neural fibers located in

the basal neural layer underneath the prostate epithelium, without affecting VAcHT⁺ parasympathetic fibers surrounding epithelial cells (fig. S4, A and B). 6OHDA-induced sympathectomy prevented the development of tumors in the prostate, suggesting a critical role for sympathetic neural activity in tumor engraftment (Fig. 1E). In control experiments, we found that 6OHDA was not toxic to tumor cells in vitro (fig. S4C), but chemically sympathectomized mice exhibited increased rates of epithelial cell apoptosis in the tumor-free prostate (fig. S4, D and E). Whereas distant metastases were detected 11 weeks after injection of PC-3luc tumor cells in control mice, no detectable metastasis was observed in sympathectomized mice, likely because of the impaired tumor development at the orthotopic site (Fig. 1E, inset). To confirm these results and to ascertain whether sympathetic signals were locally delivered in the tumor microenvironment, we surgically cut the hypogastric nerves, which carry selectively sympathetic fibers into the prostate gland, prior to orthotopic injection of tumor cells (Fig. 1F and fig. S5). Surgical denervation markedly inhibited tumor development, whereas tumors in sham-operated nerve-intact mice grew exponentially from week 4. These results suggest that SNS signals are critical at early stages of prostate tumor development in this xenogenic mouse model.

Recent studies have revealed that the SNS, a major pathway for stress-induced signals, enhances tumor growth through the β_2 -adrenergic receptor (*Adrb2*) expressed on tumor cells (7, 19) and also regulates the hematopoietic stem cell niche in the bone marrow via *Adrb2* and *Adrb3* expressed in the stroma (20, 21). On the basis of possible parallels between the behavior of the hematopoietic and cancer stem cell niches, we investigated the role of adrenergic signals in tumor engraftment by crossing *nu/nu* mice with mice that were genetically deficient in the β_2 -, β_3 -, or both β_2 - and β_3 -adrenergic receptors. Whereas tumor development in the prostate was slightly delayed in mice lacking a single adrenergic receptor, it was severely compromised in *Adrb2*^{−/−}*Adrb3*^{−/−} mice (Fig. 1G). In addition, tumor cell dissemination into the lymph nodes and distant organs was significantly reduced in the double knockouts (Fig. 1H). Although β -blockers have been suggested to inhibit metastases by antagonizing *Adrb2* expressed by tumor cells (19), we speculate that the reduced tumor volume at the orthotopic site may explain the reduction in metastasis in the *Adrb2*^{−/−}*Adrb3*^{−/−} mice. Control experiments revealed that these observations were not specific to PC-3luc cells, as tumor development and dissemination were also impaired when LNCaP human prostate cancer cells were injected into the *Adrb2*^{−/−}*Adrb3*^{−/−} mice (fig. S6). Thus, expression of both the *Adrb2* and *Adrb3* receptors in the microenvironment appears to be critical for tumor development in this mouse model.

To explore the role of sympathetic innervation in tumorigenesis in a genetic model of prostate cancer, we evaluated the effect of chemical or surgical

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sympathectomy on tumor progression in Hi-Myc transgenic mice (22). These mice selectively over-express cMyc in the prostate (under the control of the probasin promoter), which leads to the complete penetrance of mouse prostatic intraepithelial neoplasia (PIN) from postnatal week 2, progressing to invasive adenocarcinomas within 3 to 6 months (Fig. 2, A and B). Relative to vehicle-treated control mice, the incidence of PIN was reduced by 83% in mice that had been treated as 2-day-old neonates by chemical sympathectomy ($P < 0.001$; Fig. 2C). Similar results were observed after chemical or surgical sympathectomy of young adult (1-month-old) mice, although the reduction in PIN incidence was only ~25%. Sympathectomy had no effect on tumorigenesis when it was performed on Hi-Myc mice that were 2 months of age or older (Fig. 2C). Tumor invasion was also not affected by ablation of the SNS (Fig. 2D). Consistent with a role of adrenergic signals in PIN formation, immunostaining of prostate sections from tumor-bearing Hi-Myc mice revealed that adrenergic

fibers develop predominantly around healthy or PIN acini, with fewer sympathetic nerves near the tumor (fig. S7). One month after surgical sympathectomy of 1-month-old mice, we observed a marked increase in the number of apoptotic neoplastic epithelial cells in PIN (Fig. 2E). Together, these data support the notion that adrenergic signals play an important role in prostate tumorigenesis.

Regulation of Tumor Invasion by Cholinergic Parasympathetic Signaling

Because we detected the infiltration of VACHT⁺ fibers from the parasympathetic nervous system (PNS) in a subset of prostate tumors from animals that exhibited metastases, we next evaluated the possible role of the PNS in cancer progression. Post-ganglionic PNS neurons activate muscarinic cholinergic receptors on the effector organ (23). We thus profiled the expression of the five known muscarinic receptor genes (24) in the mouse prostate gland and human prostate cancer cell lines. PC-3 cells largely express *Chrm3* (cholinergic receptor, muscarinic 3),

whereas *Chrm5* was the predominant receptor expressed in DU145 and LNCaP cells (fig. S8 and table S1). Acetylcholine was previously reported to induce proliferation of prostate cancer cell lines in vitro via *Chrm3* (25). In contrast, consistent with prior studies within human prostate tissue (26, 27), we found that *Chrm1* was expressed at high levels in the healthy prostate gland of mice (fig. S8).

Accordingly, we explored whether parasympathetic activity in tumors might affect tumor progression through *Chrm1* expressed in the prostate tumor microenvironment. To investigate this, we orthotopically treated PC-3luc tumor-bearing mice with carbamoylcholine chloride (carbachol), a non-selective *Chrm* agonist, and tested the role of cognate muscarinic receptors with pharmacological antagonists. Carbachol treatment significantly enhanced tumor cell invasion of pelvic lymph nodes that drain the prostate gland (Fig. 3A and fig. S9). Local tumor cell dissemination was mediated by a muscarinic receptor, because this phenomenon was inhibited by a nonselective muscarinic

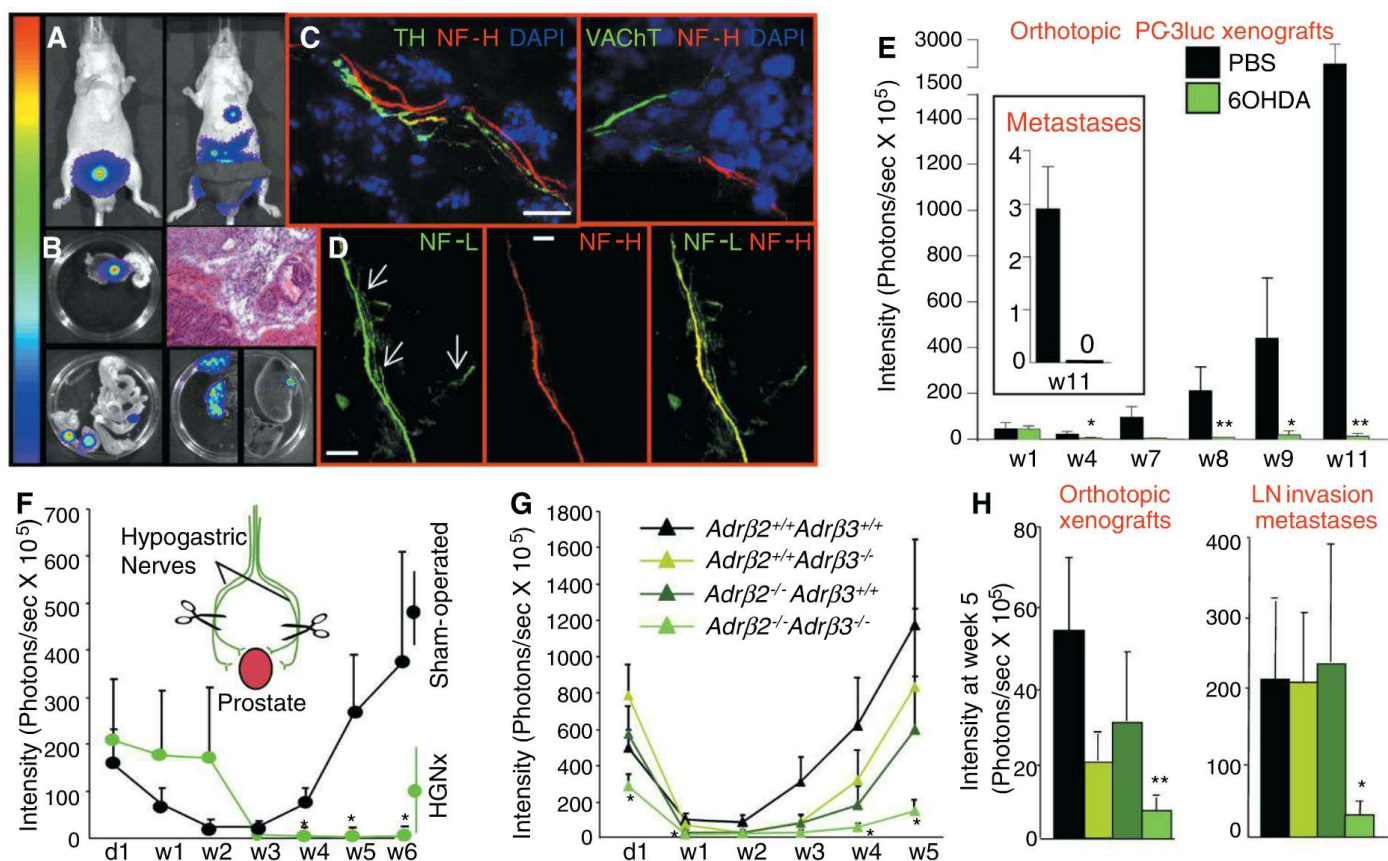


Fig. 1. Sympathetic nervous system (SNS) controls tumor engraftment in mice. (A) Examples of in vivo bioluminescence imaging of Balb/c *nu/nu* males mice 11 weeks after injection of PC-3luc cells into the ventral prostate without development of metastasis (left) and with distant metastases (right). (B) Top left: Ex vivo imaging of the prostate tumor. Top right: Representative H&E-stained section showing the intracapsular tumor surrounded by healthy prostate tissues. Bottom, left to right: Metastases within the intestine, lung, and liver. (C) Representative immunofluorescence staining of TH (left) and VACHT (right). (D) NF-L tumor nerves in green costained with NF-H (DAPI, blue; NF-H, red). Note the developing NF-L⁺ branches (arrows) arising from a double-positive fiber. (E) Serial quantification of bioluminescence intensities in tumors within the prostate,

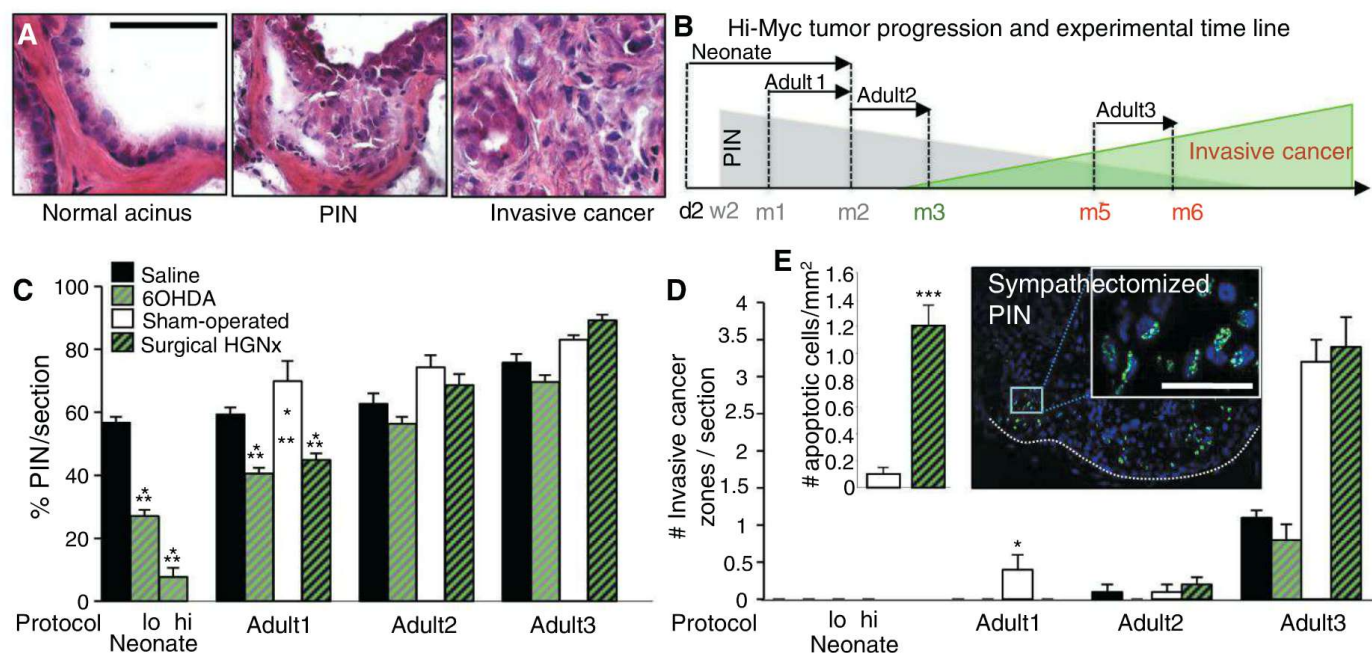
weeks 1 to 11, after xenografting in mice denervated with 6OHDA ($n = 8$) and in a PBS-treated group ($n = 11$). Inset shows quantification of pelvic lymph node invasion and metastases at week 11. (F) Real-time quantification of bioluminescence of PC-3luc tumor cells injected into prostate glands denervated of hypogastric nerves (HGNx; $n = 5$) or sham-operated ($n = 8$). (G) Serial in vivo bioluminescence analyses evaluating the growth of PC-3luc cells in the prostate of *Adrb2*^{-/-} ($n = 10$), *Adrb3*^{-/-} ($n = 10$), and *Adrb2*^{-/-}*Adrb3*^{-/-} ($n = 9$) *nu/nu* mice compared to *Adrb2*^{+/+}*Adrb3*^{+/+} and *Adrb2*^{+/+}*Adrb3*^{+/+} *nu/nu* controls ($n = 24$) of the same background. (H) Ex vivo quantification of bioluminescence in PC-3luc prostate tumors (left) and in lymph nodes and distant metastases (right) of the same mice shown in (G). * $P < 0.05$, ** $P < 0.01$. Scale bars, 10 μ m. Error bars indicate SE.

antagonist (scopolamine) (Fig. 3A). Treatment with pirenzepine, a *Chrm1*-specific antagonist, also inhibited lymph node invasion (Fig. 3A). Cardiovascular hemodynamics were not significantly altered in mice treated with carbachol (table S2), suggesting that the effects on tumor behavior were

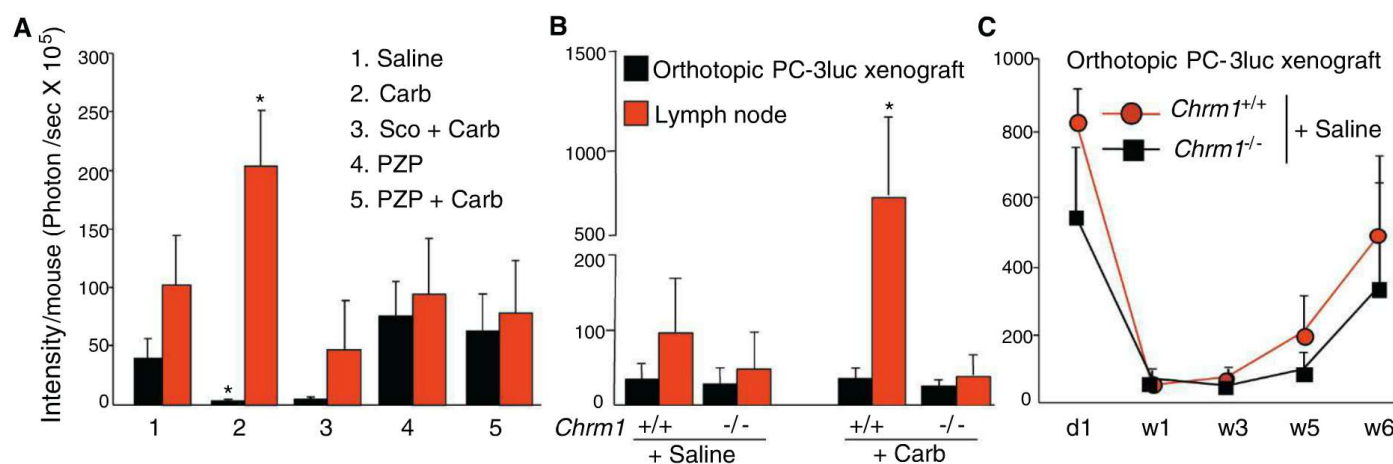
not due to nonspecific cardiovascular alterations. Tumors from carbachol-treated mice exhibited higher proliferation (Ki-67⁺) indexes, but in vitro treatment of PC-3luc cells with carbachol did not stimulate proliferation, which suggests that the effect was not tumor cell-autonomous (fig. S10).

Contribution of Stromal *Chrm1* in Tumor Invasion and Metastasis

To explore whether tumor cholinergic signals were mediated by stromal *Chrm1* expression, we crossed *Chrm1*^{-/-} mice with *nu/nu* mice and implanted PC-3luc cells into the prostate of the



birth, respectively). (C and D) Effect of systemic (6OHDA, $n = 19$ neonates, $n = 26$ adults) or local HGNx ($n = 11$) denervation of the SNS on the prevalence of PIN (C) or invasive cancer zones (D) in Hi-Myc transgenic mice. Denervation of 2-day-old neonates or at 1 month after birth, but not later, significantly reduced the percentage of PIN. Data were analyzed from 10 sections per animal. (E) Left: Quantification of apoptotic TUNEL⁺ cells in HGNx mice versus controls 1 month after surgery. Right: Illustrative TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling) of apoptotic cells in PIN from a surgically sympathectomized mouse. Data were obtained from five fields per animal ($n = 6$). * $P < 0.05$, *** $P < 0.001$. Scale bars, 10 μ m. Error bars indicate SE.



nu/nu Chrm1^{-/-} and *nu/nu Chrm1^{+/+}* progeny. Carbachol-induced tumor cell spreading to lymph nodes was markedly reduced when the prostate microenvironment was deprived of Chrm1 (Fig. 3B). Deficiency in Chrm1 signaling did not affect tumor growth at the implantation site within the prostate (Fig. 3C). This, combined with the increased proliferation of tumor cells in the prostate (fig. S10B), suggests that Chrm1 may affect the dissemination of proliferative tumor cells.

To evaluate the effect of cholinergic agonists on prostate cancer dissemination, we assessed Hi-Myc transgenic mice deficient or sufficient in *Chrm1* expression (Fig. 4, A and B). Carbachol treatment of 3-month-old transgenic mice significantly increased the incidence of PIN (Fig. 4C, left) and accelerated the progression of these neoplastic lesions to invasive carcinoma 1 month later (Fig. 4C, right). In addition, carbachol treatment significantly increased the tumor proliferative index (Fig. 4D).

Treatment of Hi-Myc mice with pirenzepine or genetic deletion of *Chrm1* completely inhibited carbachol-induced malignant progression (Fig. 4, C and D). To confirm the role of stromal *Chrm1* expression in prostate cancer progression, we implanted c-Myc⁺ *Chrm1^{-/-}* prostate acini into the dorsal lobe of healthy *Chrm1^{+/+}* nude mice. In the resulting chimeric prostate, only the donor prostate tissue could develop cancer and only the recipient tissue could respond to Chrm1-mediated signals (Fig. 4E, left box). Treatment of engrafted mice with carbachol switched the tumor behavior to an invasive phenotype with stromal Chrm1-mediated disruption of the basement membrane and proliferation of tumor epithelial cells (Fig. 4E, top row). Thus, these studies demonstrate that cholinergic signals transduced in the tumor stroma by the type 1 muscarinic receptor promote prostate cancer invasion in two mouse models of prostate cancer.

Ex vivo bioluminescence quantification of metastases to distant organs (see Fig. 1B) also revealed a factor of ~6 increase in tumor cell dissemination in carbachol-treated mice relative to the control group (Fig. 5, A to C). Metastases were reduced in mice in which type 1 muscarinic receptor function was pharmacologically or genetically ablated (Fig. 5, A to C), leading to improved survival of the animals (Fig. 5, D and E). We confirmed these results in older (18- to 24-month-old) Hi-Myc transgenic mice. Positron emission tomography (PET) imaging using [¹⁸F]fluoro-2-deoxy-D-glucose (¹⁸FDG) showed significantly more spontaneous soft tissue metastases in c-Myc⁺ *Chrm1^{-/-}* mice relative to c-Myc⁺ *Chrm1^{+/+}* animals (Fig. 6, A and B). By contrast, FDG uptake in primary tumor sites did not differ between the two strains (Fig. 6, A and B). Moreover, bone metastases traced by [¹⁸F]sodium fluoride (Na¹⁸F) were detected in c-Myc⁺ *Chrm1^{+/+}* mice but not in Hi-Myc mice

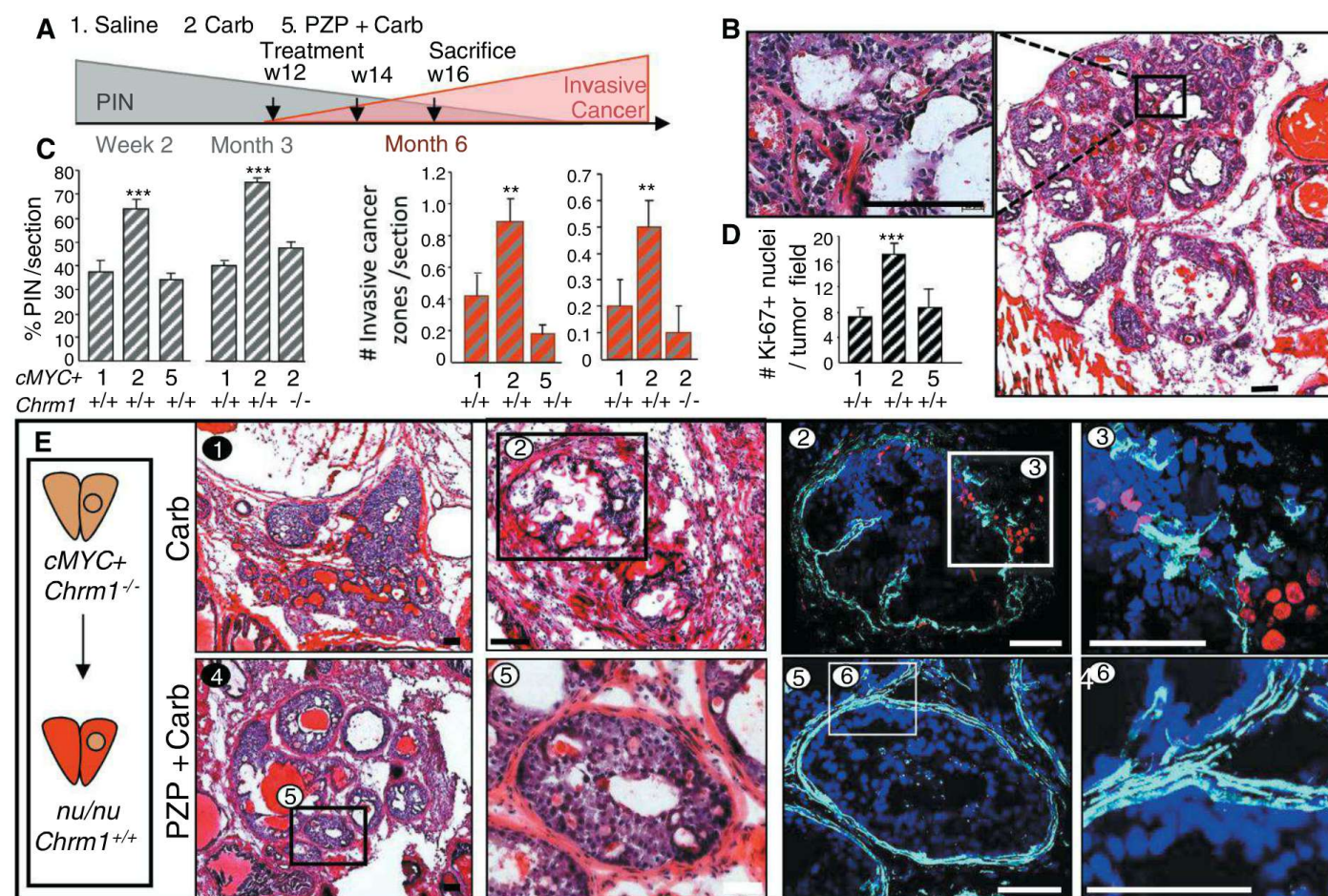


Fig. 4. Chrm1 promotes prostate cancer progression in Hi-Myc transgenic mice. (A) Timeline for Hi-Myc-induced tumor progression and therapeutic schedule. (B) H&E-stained section of the prostate from an animal treated by carbachol; boxed area shows higher magnification of the invasive cancer zone. (C) Percentage of neoplastic acini (left) and number of invasive cancer zones per prostate section (right) after treatment by carbachol [treatment 2 from (A)], pharmacological inhibition of Chrm1 [treatment 5 from (A)], or genetic disruption of Chrm1 in the cMyc⁺ *Chrm1^{-/-}* mice. (D) Quantification of Ki-67 staining in treatment groups 1, 2, and 5. (E) Histological analyses of prostate chimeric tissues. cMyc⁺ *Chrm1^{-/-}* prostate tissues were grafted in the dorsal lobe of *nu/nu*

Chrm1^{+/+} prostate glands (left). Tissues from mice treated with carbachol (top row) or carbachol + PZP (bottom row) were harvested 10 weeks after surgery. H&E-stained sections of cMyc⁺ *Chrm1^{-/-}* grafts show invasive cancer areas at late (1) or early (2) stage of development in the carbachol-treated group. PIN lesions were maintained when Chrm1 was blocked by PZP prior to carbachol injection (4, 5). Immunofluorescence staining for laminin- α 2 (light blue) shows disruption of basement membranes (2, 3) and Ki-67⁺ (red) cancer cells (3) in carbachol-treated chimeras relative to the intact basement membrane in the PZP + carbachol-treated group (5, 6) (DAPI, dark blue). ***P* < 0.01, ****P* < 0.001. Scale bars, 100 μ m [(B) and (E)]. Error bars indicate SE.

lacking *Chrm1* (Fig. 6, C to E). These data suggest that muscarinic signals promote cancer metastasis.

Autonomic Neural Network in Human Prostate Cancer

To evaluate the relevance of autonomic innervation in human cancer, we retrospectively analyzed nerve fiber densities in prostatectomy tissues from 43 treatment-naïve patients with prostate cancer. These patients had low-risk cancer [prostate-specific antigen (PSA) level <10 ng/ml, Gleason score <7, and disease stage T1c or T2a; $n = 30$] or high-risk cancer [PSA level ≥ 10 ng/ml, or Gleason score ≥ 7 , or disease stage $\geq T2b$; $n = 13$] (28, 29), and they had been treated at the VA Medical Center in Durham, North Carolina (Fig. 7 and table S3). Quantification of nerve fiber densities (TH⁺, VACHT⁺, NF-L⁺, and NF-H⁺) was performed in a blinded fashion without prior knowledge of clinical or pathological stage and clinical outcome. Nerve-specific staining for NF-L or NF-H revealed increased fiber densities within tumor areas, and also in normal prostate tissues surrounding the cancer in high-risk patients (fig. S11). Further individual assessment of each branch of the autonomic nervous system showed that TH⁺ adrenergic fibers densely innervated normal prostate tissues surrounding the tumor (Fig. 7, A to C), whereas VACHT⁺ cholinergic fibers were largely restricted to the

tumor (Fig. 7, D to F). In addition, high nerve densities were associated with a higher tumor proliferative index [Fig. 8A and fig. S12; Pearson correlation coefficients = 0.529 and 0.649 for TH and VACHT, respectively ($P < 0.001$)]. These data are consistent with an aberrant, but orchestrated, recruitment of sympathetic and parasympathetic nerve fibers related to the malignancy.

We next assessed whether there was a relationship between nerve density and the clinical progression of the disease. The number of TH⁺ fibers in normal areas or VACHT⁺ fibers in cancer was found to associate positively with preoperative levels of PSA, as determined by a linear regression model ($P = 0.0007$ and $P = 0.02$ for TH and VACHT, respectively). Bivariate association revealed that TH⁺ nerve density and overall nerve densities of the normal tissue were positively correlated with time to biochemical recurrence ($P = 0.0039$, $P = 0.0095$, and $P = 0.0454$ for NF-L, NF-H, and TH, respectively) and to tumor spreading outside of the prostate ($P < 0.0001$ for each). In addition, VACHT⁺, NF-L⁺, and NF-H⁺ fibers in cancer were significantly associated with extra-prostatic extension ($P < 0.0001$ for each). Cox and logistic regression models confirmed these associations (tables S4 and S5). Statistical significance associated with fiber densities was not sustained in multivariable analysis when clinical variables including PSA levels and Gleason score were

adjusted. This finding may relate to the association of fiber densities with these clinical variables and/or the small sample size that limits the possibility of detecting the independent prognostic value of the biomarkers. However, specific thresholds of TH⁺ nerve areas per field of normal tissue ($>2000 \mu\text{m}^2$ per field; Fig. 8B) or VACHT⁺ nerve areas in tumor tissue ($>300 \mu\text{m}^2$ per field; Fig. 8B) at diagnosis were associated with higher recurrence rates. These preliminary results suggest that nerve density assessment merits exploration as a possible predictive marker of prostate cancer aggressiveness (28).

Discussion

Prior studies have described a process termed perineural invasion in which tumor cells grow and migrate along native nerve fibers. This process is associated with a poor prognosis, possibly because the nerves provide survival signals to the tumors (30) or provide a gateway toward hematogenous spread (31). The present results uncover a distinct phenomenon, possibly analogous to angiogenesis (16), where the tumor itself is infiltrated by a network of newly developed autonomic nerve projections that regulate cancer initiation and progression.

Studying mouse models, we have found complementary functions for the two branches of the autonomic nervous system: Adrenergic fibers from the SNS, acting through stromal β_2 - and

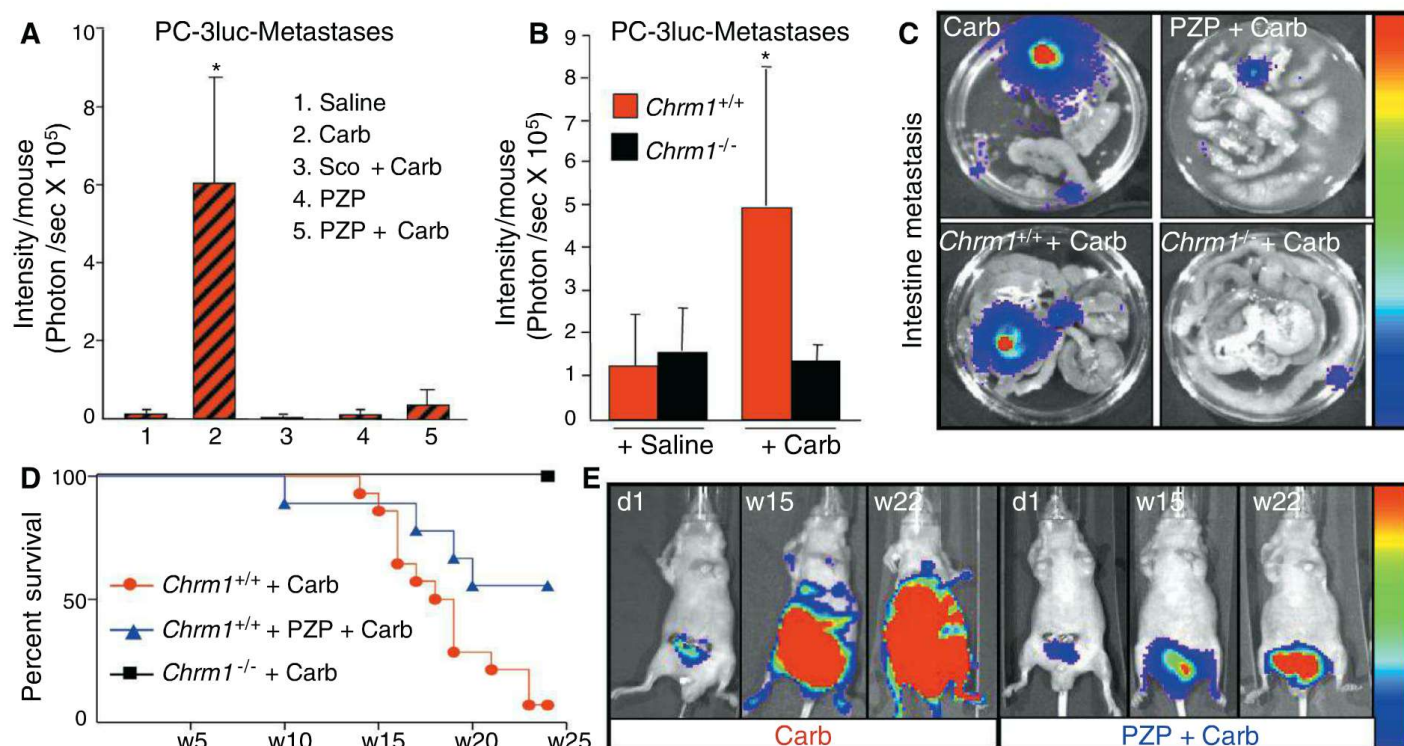


Fig. 5. Cholinergic signal contributes to prostate cancer metastasis in xenografted mice through the type 1 muscarinic receptor *Chrm1*. (A and B) Ex vivo quantification of bioluminescence from distant organs at week 5 after selective pharmacologic (A) or genetic (B) inactivation of *Chrm1*; $n = 7$ mice per group. (C) Representative ex vivo bioluminescence imaging of intestinal metastases induced by carbachol with (right) or

without (left) *Chrm1* inhibition. (D) Kaplan-Meier curves depicting the survival of metastatic *nu/nu Chrm1*^{+/+} mice treated with carbachol ($n = 14$) by comparison to nonmetastatic carbachol-treated *nu/nu Chrm1*^{-/-} animals ($n = 6$; $P = 0.0005$, log-rank test) or *nu/nu Chrm1*^{+/+} mice treated with PZP + carbachol ($n = 9$; $P = 0.03$, log-rank test). (E) Representative images of mice from (D) at different time points. $*P < 0.05$. Error bars indicate SE.

β_3 -adrenergic receptors, play an important role in the initial phases of cancer development by promoting tumor cell survival, while cholinergic fibers of the PNS play predominant roles in tumor cell invasion, migration, and distant metastases through stromal *Chrm1*-mediated signals (Fig. 8C). This idea is supported by the innervation patterns of human prostate cancer, where sympathetic fibers accumulate in normal tissues and infiltrate the tumor edge, whereas parasympathetic fibers frankly infiltrate tumor tissues. These results are consistent with recent epidemiological data suggesting that β -blocker intake is associated with improved survival of prostate cancer patients (13, 14). It is notable that clinically used β -blockers

are largely selective to the β_1 adrenoceptor, and thus our results suggest that these clinical studies may underestimate their potential benefits. Although further studies will be required to dissect the molecular events linking tumor “neurogenesis” to cancer progression, our data raise the tantalizing possibility that drugs targeting both branches of the autonomic nervous system may be useful therapeutics for prostate cancer.

Materials and Methods

Balb/c *nu/nu* (B6.Cg-*Foxn1*^{nu/+}) and Hi-Myc mice [FVB-Tg(ARR2/Pbsn-MYC)7Key (22)] were obtained from Charles River laboratories and the National Cancer Institute, respectively.

Balb/c heterozygous nude mice were intercrossed with *Adrb2*^{tm1Bkl/J-/-} and *Adrb3*^{tm1Low/J-/-} mice or with *Chrm1*^{tm1Sit-/-} mice obtained from the Jackson Laboratory. FVB-Tg(ARR2/Pbsn-MYC)7Key *Chrm1*^{tm1Sit-/-} and respective controls were also generated by intercrossing the two strains.

All in vivo experiments were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine and Albert Einstein College of Medicine. Human tumors were induced by orthotopic surgical implantations of 10⁵ PC-3luc cells into 6- to 8-week-old Balb/c *nu/nu* mice. Ten days after cell injection (day 0), the animals were randomized into the different groups and received the appropriate drugs as indicated. 6OHDA

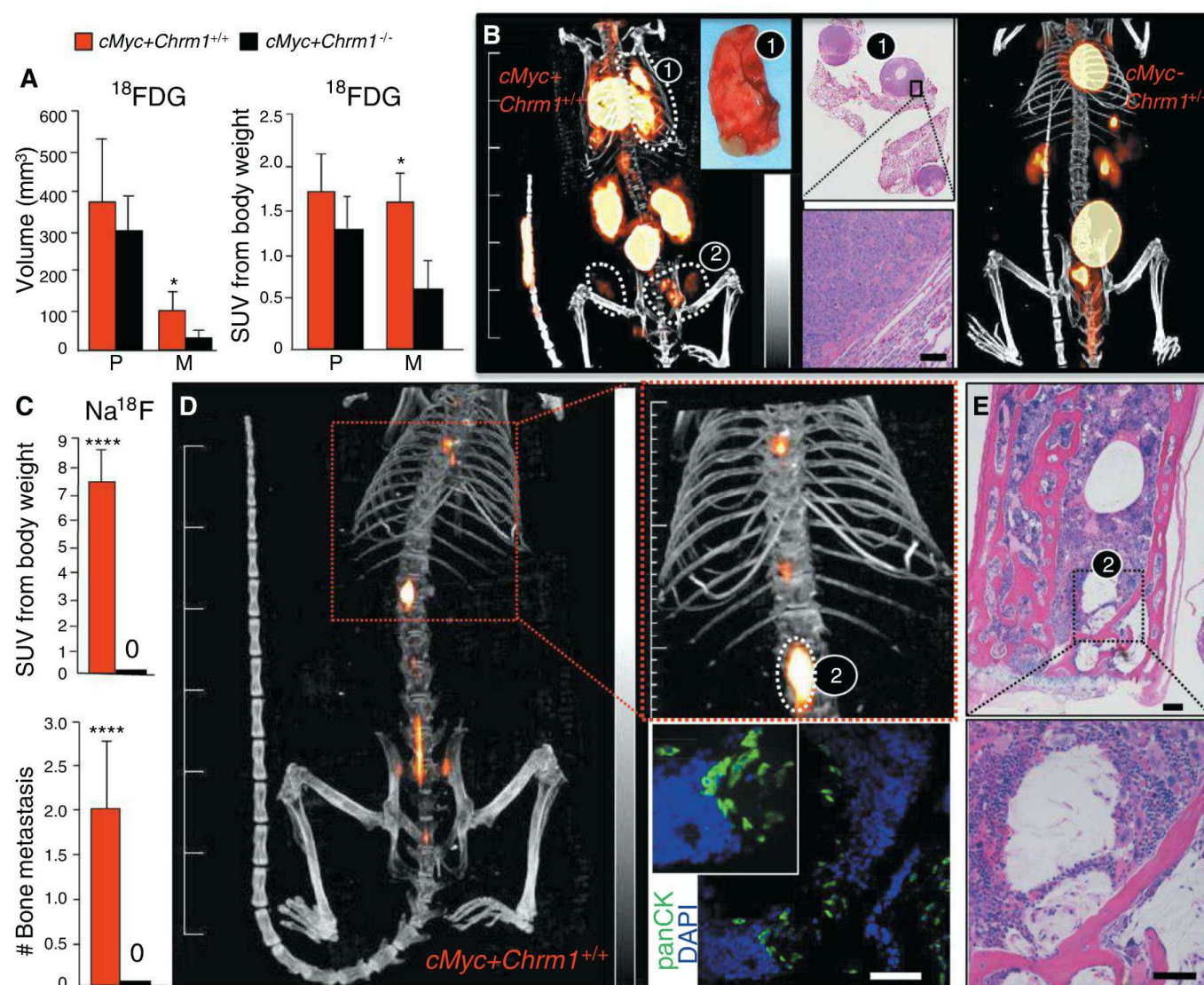


Fig. 6. *Chrm1* controls prostate cancer metastasis in Hi-Myc transgenic mice. (A) Tumor volume (left) and maximum standard uptake value (SUV_{max}) (right) of ¹⁸FDG⁺ tumors in the prostate (P) or metastases (M) to lung or in paraspinal lymph nodes obtained by PET scanning of 18- to 24-month-old cMyc⁺ *Chrm1*^{+/+} or cMyc⁺ *Chrm1*^{-/-} mice; *n* = 5 to 10 mice per group. (B) ¹⁸FDG⁺ images of a cMyc⁺ *Chrm1*^{+/+} cancer-bearing mouse (left) with abnormal soft-tissue pelvic uptake (dotted area 2) and lung uptake (dotted area 1, with necropsy photograph confirming spontaneous lung metastases and matched H&E-stained section with boxed area at higher magnification). Note the absence of ¹⁸FDG⁺ spots in the lung or pelvis of a control cMyc⁻

Chrm1^{+/+} wild-type mouse (right). (C) SUV_{max} (top) and number of bone metastases (bottom) in spine quantified by Na¹⁸F-PET scanning. (D) Representative images of a bone metastatic cMyc⁺ *Chrm1*^{+/+} mouse (left) injected with Na¹⁸F; boxed area shows higher magnification of a front view of thorax (right, dotted red box). (E) H&E-stained section of T12/L1 vertebrae (top) of the boxed area 2 shown in (D); higher magnification (bottom) shows prostate tumor metastasis in bone confirmed by consecutive pan-cytokeratin (panCK)-stained section of epithelial cells in green (DAPI, blue). **P* < 0.05, *****P* < 0.0001. Scale bars, 50 μ m for H&E and fluorescence images, 10 mm [(B) and (D), left] and 1 mm [(D), right] per interval for PET images. Error bars indicate SE.

or vehicle was injected at day 0 (100 mg/kg) and day 2 (250 mg/kg). In other experiments, 2×10^5 PC-3luc cells were orthotopically injected into *nu/nu* *Adrβ2^{tm1Bkk/J+/-}* *Adrβ3^{tm1Low/J+/-}*, *nu/nu* *Adrβ2^{tm1Bkk/J-/-}*, *nu/nu* *Adrβ3^{tm1Low/J-/-}*, and *nu/nu* *Adrβ2^{tm1Bkk/J-/-}* *Adrβ3^{tm1Low/J-/-}* mice. In selected experiments, 2×10^6 LNCaP-luc cells were injected in *nu/nu* *Adrβ2^{tm1Bkk/J+/-}* *Adrβ3^{tm1Low/J+/-}* and *nu/nu* *Adrβ2^{tm1Bkk/J-/-}* *Adrβ3^{tm1Low/J-/-}* mice. For the transgenic model, 1-, 2-, and 5-month-old Hi-Myc mice (Adult1, Adult2, and Adult3, respectively) were injected with 6OHDA or surgically sympathectomized according to protocols described above and killed 30 days later. Neonates Hi-Myc littermates were injected with 6OHDA at day 2 (lo, 100 mg/kg; hi, 200 mg/kg), day 4 and 6 (lo, 100 mg/kg; hi, 400 mg/kg), at day 8 (lo, 100 mg/kg; hi, 800 mg/kg) and at day

9 (lo, 100 mg/kg) after birth and killed 30 or 60 days later.

For experiments on the PNS, 15 days after tumor cell injection, animals received carbamoylcholine chloride (Sigma) at 250 (day 0), 300 (day 1), 350 (day 2), 500 μg/kg per day (day 3) [every 12 hours, 8 divided doses intraperitoneally (i.p.) in saline] alone or in combination with scopolamine hydrobromide (Sigma, 1 mg/kg) (32) or pirenzepine dihydrochloride (Sigma, 6 mg/kg) (33). A second cycle was administered at week 4 and mice were killed 1 week later (5 weeks after graft). For experiments using *nu/nu* *Chrm1^{tm1Std+/+}* and *nu/nu* *Chrm1^{tm1Std-/-}* animals, mice were killed at week 6 after grafting. For survival study, grafted mice were treated as described above from week 4, every 2 weeks for up to 25 weeks or until death. Disease progression was monitored by bioluminescence

scanner. For Hi-Myc model, 3-month-old mice were injected with carbachol alone or in combination with pirenzepine for 4 days after the protocol described above. One week later, mice received a second round of treatment and then were killed at month 4. For cMyc⁺ acini implantation, 2-month-old cMyc⁺ *Chrm1^{tm1Std-/-}* acini were implanted into 6-week-old *nu/nu* *Chrm1^{tm1Std+/+}* recipients. After 5 weeks, mice were treated with two cycles (week 6 and 8) of carbamoylcholine chloride alone or combined with pirenzepine dihydrochloride as described above. Mice were killed at week 10.

Cell Culture

PC-3 cells stably transfected with the luciferase gene (PC-3luc, gift from J. Blanco, CSIC, Barcelona, Spain) were grown in F12-Glutamax medium supplemented with 10% fetal bovine

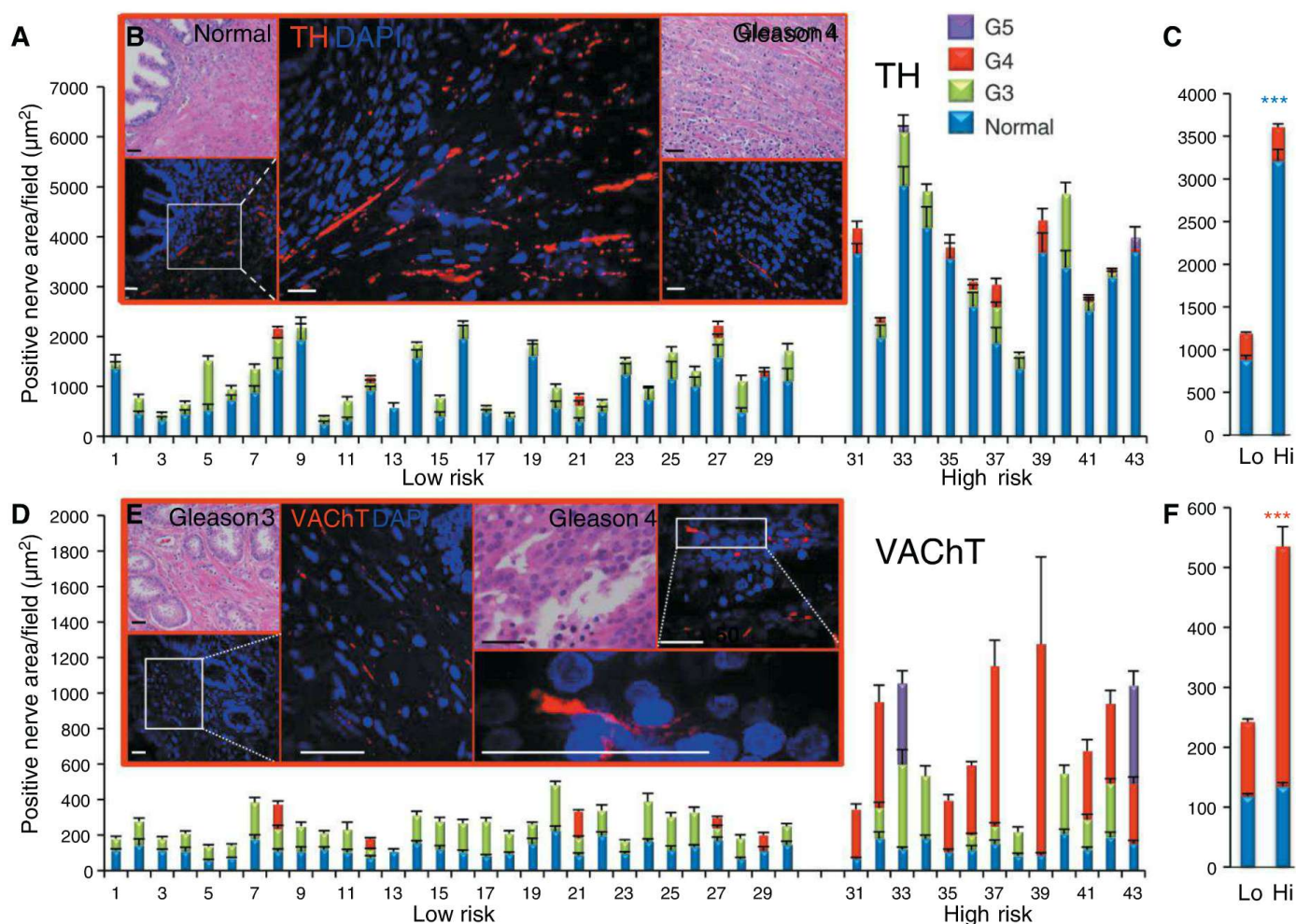


Fig. 7. Human high-risk prostate adenocarcinomas are rich in adrenergic and cholinergic nerve fibers. (A) Quantification of immunostained TH⁺ neural areas in low-risk ($n = 30$) and high-risk ($n = 13$) human prostate adenocarcinomas. Representation of TH⁺ nerve densities per field in normal and Gleason grade 3, 4, or 5 tumor areas. Each bar represents the averages for a patient. (B) Representative images showing the thick disorganized TH⁺ adrenergic neural network in normal tissues surrounding cancer in a high-risk patient (left, TH⁺, red; DAPI⁺, blue; with matched H&E section) and boxed area showing higher magnification of infiltrating fibers. By contrast, Gleason 4 invasive adenocarcinoma displays fewer discrete nerves (right).

(C) Average TH⁺ fiber densities in both normal (blue) and cancer (red) tissues of low-risk (Lo) and high-risk (Hi) patients. (D) Quantification of cholinergic VACHT⁺ nerves in normal and tumor tissues in the same patients as in (A). (E) Immunofluorescence images of VACHT⁺ nerves (red; DAPI⁺, blue) in Gleason 3 (left) and Gleason 4 (right) tumor areas, with matched H&E-stained fields and boxed areas showing higher magnification. (F) Average VACHT⁺ fiber densities compiled as in (C). For (A) and (D), each bar represents average nerve densities of a patient obtained from 10 fields per Gleason grade or per normal area, field surface = 0.15 mm². Scale bars, 50 μm. *** $P < 0.001$. Error bars indicate SE.

serum (FBS), bicarbonate sodium (1.5 g/liter), and G418 (500 mg/ml; Invitrogen). LNCaP cells expressing luciferase (Xenogen; Caliper Life Sciences, Hopkinton, MA) were grown according to manufacturer's recommendations in RPMI medium (ATCC#30-2001) supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco).

Bioluminescence Imaging

In vivo and ex vivo bioluminescence imaging was performed and analyzed using an IVIS imaging system 200 series (Xenogen). Bioluminescent signal was induced by i.p. injection of D-luciferin [150 mg/kg in phosphate-buffered saline (PBS)] 8 min before in vivo imaging. For ex vivo imaging, D-luciferin (300 mg/kg) was injected 7 min

before necropsy. Organs of interest were immersed in a solution of D-luciferin at 150 mg/ml (17).

Histology and Immunofluorescence

Upon killing, mouse prostate tissues were immersed in OCT medium. Frozen sections (thickness 5 μ m) were stained with hematoxylin and eosin (H&E). For immunofluorescence, prostate sections were fixed with acetone or methanol and incubated in H₂O₂ to quench endogenous peroxidase. Nonspecific binding was blocked with goat serum in BSA solution and an avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) (20). Sections were incubated with a rabbit antibody to TH (Millipore, Billerica, MA), or antibody to VAcHT (Phoenix Pharmaceuticals, Burlingame,

CA), or antibodies to NF-L (Millipore) or NF-H (Abcam, Cambridge, MA) followed by secondary biotinylated goat antibody to rabbit immunoglobulin G (IgG) (Vector). Signal was amplified by Vectastain Elite ABC Kit (Vector) and visualized by Tyramide Signal Amplification kit for TRITC (PerkinElmer). For proliferative cell quantification, sections were incubated with a rabbit polyclonal antibody to Ki-67 (Abcam) and then Alexafluor568-conjugated goat antibody to rabbit IgG (Molecular Probes). For apoptotic cell quantification, prostate sections were fixed with 4% paraformaldehyde and stained with the Mebstain Apoptosis Kit according to manufacturer's recommendations (MBL International, Woburn, MA). Basement membranes were stained with a rat polyclonal antibody to α 2laminin

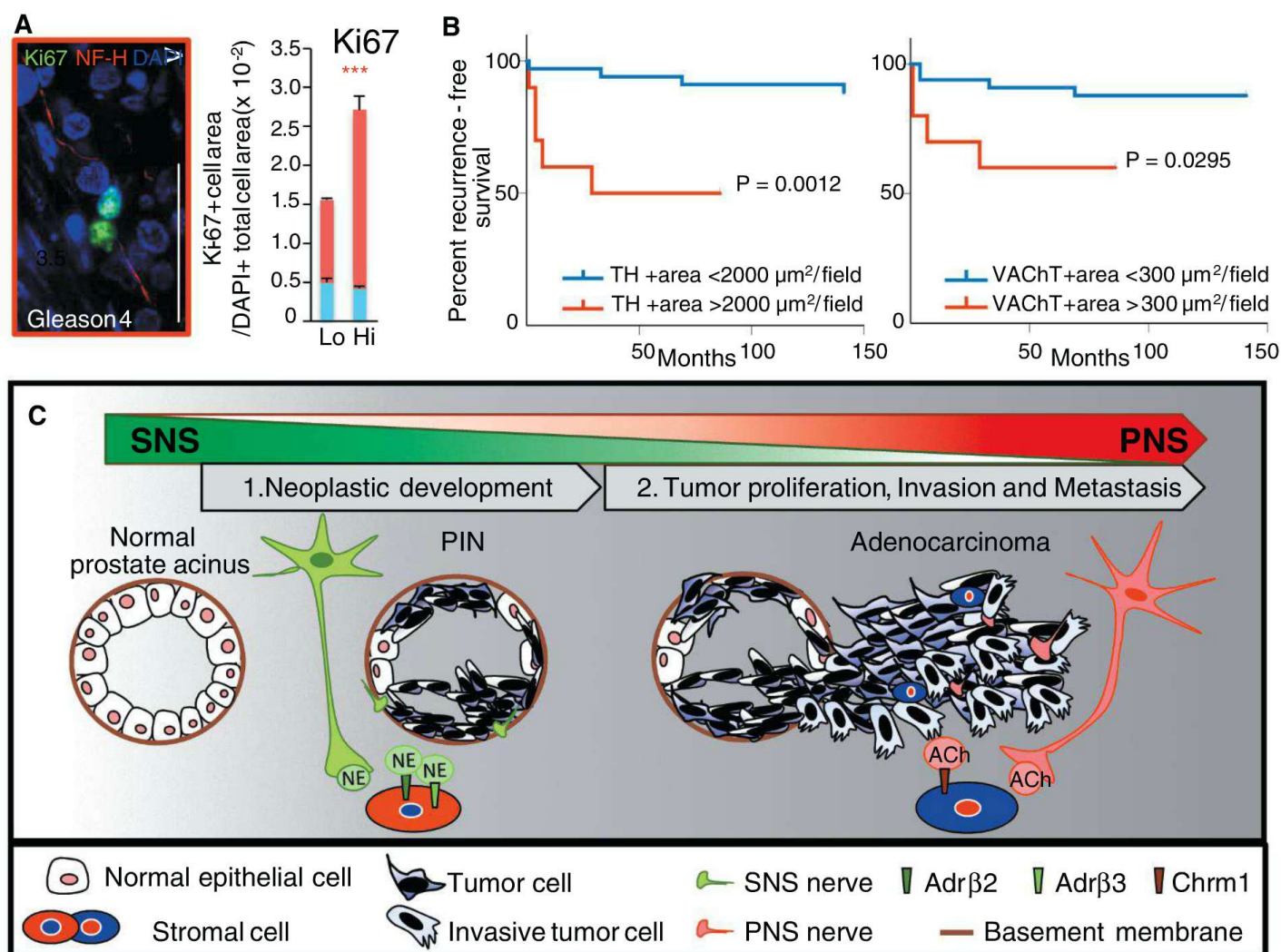


Fig. 8. Density of nerve fibers in human prostate cancer specimens correlates with tumor aggressiveness. (A) Left: Representative immunofluorescence image of Ki-67⁺ nuclei (green; DAPI⁺, blue; NF-H⁺ fibers, red) in a Gleason 4 cancer area. Right: Average proliferative indexes in normal (blue) or cancer (red) areas. Bars represent average proliferative indexes obtained from 10 fields per Gleason grade or per normal area per patient, field surface = 0.15 mm². Scale bar, 50 μ m. $***P < 0.001$. (B) Left: Recurrence-free survival of patients with high (>2000 $\mu\text{m}^2/\text{field}$) and low (<2000 $\mu\text{m}^2/\text{field}$) adrenergic nerve densities. Right: Recurrence-free survival of patients with high (>300 $\mu\text{m}^2/\text{field}$) and low (<300 $\mu\text{m}^2/\text{field}$)

cholinergic nerve densities. Error bars indicate SE. (C) Schematic illustration showing how prostate cancer initiation and metastasis may be regulated by dual neural mechanisms. Nerve fibers from the sympathetic nervous system (SNS) deliver norepinephrine (NE) from nerve terminals, which acts on β_2 - and β_3 -adrenergic receptors (Adr β_2 , ADR β_3) expressed on stromal cells, promoting the survival of cancer cells and the initial development of the tumor. Nerve fibers from the parasympathetic nervous system (PNS) also invade tumors, delivering acetylcholine (ACh), which promotes tumor proliferation and egress to lymph nodes and distant organs through the type 1 muscarinic receptor (Chrm1) expressed on stromal cells.

(Abcam) followed by Alexafluor647-conjugated goat antibody to rat IgG (Molecular Probes). For bone staining, samples were fixed and decalcified prior to embedding in paraffin. Bone sections were stained with a pancytokeratin antibody from Sigma. Human prostate tissues were previously fixed and embedded in paraffin as part of routine care at the Durham VA Medical Center. Blocks were serially sectioned (thickness 5 μ m) and H&E staining was performed using standard procedures. For immunofluorescence analyses, sections were deparaffinized with xylene and rehydrated through graded alcohol washes followed by antigen retrieval in sodium citrate buffer following manufacturer recommendations (Vector). Primary antibodies (rabbit antibody to TH, chicken antibody to NF-H, rabbit antibody to NF-L from Millipore, rabbit antibody to VACHT from MBL International) were incubated overnight, followed by amplification steps only for TH and VACHT stainings, as described above. For NF-L or NF-H stainings, slides were blocked in goat serum and BSA solution and then subsequently incubated with goat antibody to rabbit IgG or to chicken IgG, respectively. For proliferative index, sections were incubated with an antibody to human Ki-67 (Vector) followed by the amplification steps described above.

Bright-field images were captured and collected with a Zeiss axioplan2 microscope (Zeiss MicroImaging, Thornwood, NY) and with a Q-imaging MP3.3 RTV color camera controlled by Zeiss AxioVision software. Full Hi-Myc prostate sections were captured with a Zeiss Axioplan2IE and a Zeiss AxioCamMRc camera controlled by Zeiss AxioVision software equipped with a motorized stage that automates montage acquisition and stitching for high-resolution images of large areas.

Fluorescence images were captured and analyzed using a Axio Examiner.D1 microscope (Zeiss) equipped with a Yokogawa CSU-X1 confocal scanner head with four-stack laser system (405-, 488-, 561-, and 642-nm wavelengths). Images were obtained as three-dimensional (3D) stacks scanning through the whole thickness of the tissue using CoolSnap HQ digital camera (Roper Scientific, Munich) and analyzed using Slidebook software (Intelligent Imaging Innovations, Denver).

Human Prostate Samples

Preexisting human formalin-fixed paraffin-embedded radical prostatectomies were obtained for staining after internal review board approval by the Durham VA Medical Center. Patient characteristics including age, race, and dates of surgery are shown in table S3. All patients underwent primary radical prostatectomy and had histologically confirmed and clinically localized prostate cancer [stage T1-T2NxM0 in the tumor-node-metastasis classification system according to the American Joint Committee on Cancer (34)]. For each patient, data on the primary tumor (clinical and pathological stages and Gleason grade) were recorded, as well as preoperative PSA levels. PSA recurrence was defined as a single PSA value at

>0.2 ng/ml, two values at 0.2 ng/ml, or secondary treatment for a rising PSA. Patients treated with adjuvant therapy with an undetectable PSA ($n = 6$) were censored as nonrecurrent at the time of adjuvant treatment. Recurrence might be local or distant, although no metastasis has been documented thus far in this cohort of patients. Median PSA follow-up among men without recurrence was 57 months. Extraprostatic extension was defined as disease involving one or more of extracapsular, bladder neck, or seminal vesicle extension. Surgically resected primary tumors were paraffin-embedded and serially sectioned (thickness 5 μ m). For each block, a section was stained with H&E to evaluate tissue viability, to localize normal areas among cancer, and to map the different Gleason grade areas. For each patient, the whole histological section was analyzed by two independent pathologists from the Durham VA Medical Center and Albert Einstein School of Medicine to define the Gleason grade. Assessment of nerve densities was conducted blind, without knowledge of histological diagnoses and clinical data. For each patient ($n = 43$), consecutive sections were stained for TH, VACHT, both NF-L and NF-H, or Ki-67 as described above to quantify adrenergic, cholinergic, and total autonomic nerve fiber densities or cell proliferation, respectively, in prostate tumor areas (Gleason grade from 3 to 5) and in remaining normal prostate tissues surrounding cancer areas. For each marker defined above, the average of 10 representative fields (one field = 0.15 mm² for Zeiss 20 $\times$ /1.0 NA objective) was calculated from normal areas and for each tumor grade (when present) captured as described above. A total of 3989 z-stack images were acquired and converted in 2D maximum projections that were digitally analyzed with the Slidebook to quantify nerve fiber areas and Ki-67⁺/DAPI⁺ cell areas per field (DAPI, 4',6-diamidino-2-phenylindole). For these analyses, masks were drawn to delimit precisely contours of nerve fibers or Ki-67⁺ or DAPI⁺ cells. The surface areas of TH⁺, VACHT⁺, NFL-L⁺, or NF-H⁺ nerve fibers and the ratios between Ki-67⁺ and DAPI⁺ cells were then established.

Positron Emission Tomography (PET) Imaging

All mice were imaged after 12 hours of fasting and anesthetized throughout the whole procedure with 1.5% isoflurane-O₂ mixture. Animals were injected with 400 μ Ci of either [¹⁸F]fluoro-2-deoxyglucose (FDG) or Na¹⁸F, in 0.1 ml of normal saline, into the tail vein. Image acquisition started 50 min after injection, using an Inveon Multimodality scanner (Siemens, Malvern, PA) with the PET module, which provides 12.7-cm axial and 10-cm transaxial active field of view. The PET scanner has no septa and acquisitions were performed in 3D list mode. A reconstructed full width at half maximum (FWHM) resolution of <1.4 mm was achievable in the center of the axial field of view. List mode acquisition of data was performed to permit dynamic re-

framing for kinetic evaluation of radiotracer uptake. After each acquisition, data were sorted into 3D sinograms, and images were reconstructed using a 2D-ordered subset expectation maximization algorithm. Data were corrected for decay, deadtime counting losses, random coincidences, and the measured nonuniformity of detector response, but not for attenuation or scatter. Analyses were performed using IRW dedicated software (Siemens). All images were inspected visually in a rotating 3D projection display to examine for interpretability and image artifact. Regions of interest were drawn around areas of pathologic uptake. Successive scrolling through 2D slices (each 1.2 mm thick in the axial images) permits measurement of a radioactivity within defined volumes. Corrected counts per cc within this volume divided by the counts per gram of total body mass of injected radioactivity determines the standardized uptake value (SUV); SUV_{max} is the maximum value of SUV within each tumor volume. The total number of bone spots uptaking Na¹⁸F has also been determined for each mouse.

Assessment of Hemodynamics and Cardiac Function

To evaluate the effect of the cholinergic agonist on blood flow, mice were anesthetized and prepared for intravital microscopy as described (35) 1 hour after carbachol injection. Centerline red blood cell velocities were measured for 10 venules and 10 arterioles in two independent experiments. Wall shear rates (γ) were calculated according to Poiseuille's law for a Newtonian fluid, $\gamma = 2.12(8V_{\text{mean}})/D_v$ or D_a , where D_v and D_a are the venular or arterial diameters, the mean blood flow velocity (V_{mean}) was estimated as $V_{\text{RBC}}/1.6$, and 2.12 is a median empirical correction factor obtained from actual velocity profiles measured in microvessels *in vivo* (35). The blood flow rate was calculated from the formula $V_{\text{mean}}\pi D^2/4$.

Cardiac function was assessed by echocardiography using the Vevo 2100 ultrasound imaging system, in which mice were treated for four consecutive days with saline or carbachol according to the protocol described above ($n = 4$ per group). For imaging, animals were anesthetized with a mixture of O₂/1.5% isoflurane and then positioned ventral side up on the platform of the imaging system. ECG signal and respiratory rate were captured through the electrode pads on the advanced physiological monitoring unit and transmitted to the Vevo system for monitoring. Cardiac examinations were performed in 2D images using the parasternal long axis (PLAX) view in B-mode with a IMS550D 40-MHz probe. Two cine-loops (300 frames per cine-loop) were recorded per animal and then analyzed on two diastoles and two systoles per animal. The endocardial stroke volume and endocardial diastolic volume were determined to calculate the ejection fraction.

Proliferation Assay

Experiments were carried out as described (36).

RNA Extraction and qRT-PCR

Gene expression levels were analyzed from RNA extracted, using TRIzol solution (Invitrogen), from PC-3, DU145, LNCaP cell lines or prostates from Balb/c *nu/nu* mice by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR), as described (20, 27). Primer sequences are shown in table S1.

Statistical Analyses

For mouse studies, all values are reported as means $\pm$ SEM. Statistical significance for three or more groups was assessed by a nonparametric one-way analysis of variance (Kruskal-Wallis), followed by an unpaired Mann-Whitney test. Significance was set at $P < 0.05$. The Kaplan-Meier method was used for survival curve analysis, and the log-rank (Mantel-Cox) test was used to determine the statistical significance of difference between survival curves using Graphpad Prism 5 software.

For human studies, we examined bivariate associations between each of the markers and the outcome variables. For biochemical tumor recurrence and extraprostatic extension (yes or no), a Wilcoxon ranked sum test was used. Pearson correlations between markers were calculated to examine the association with proliferative indexes. The association between TH or VACHT expression and time to recurrence was determined using a Kaplan-Meier plot and statistical significance assessed by log-rank test. Regression models were used to further estimate the magnitude of the association: A linear regression model was used to estimate the association of nerve densities in normal tissue and cancer separately as well as jointly with preoperative PSA levels; a Cox proportional-hazards model was used to estimate hazard ratios with 95% confidence intervals (CIs) for the association between the same predictors with time to biochemical tumor recurrence; and a logistic regression model was used to estimate odds ratios and 95% CIs for the association between the same predictors with tumor invasion (yes or no). Several steps of multivariate analysis were also conducted in the regression models with initial adjustments for age and/or race (characterized as black versus otherwise) and then PSA score and Gleason stage. Significance level was set at $P < 0.05$. Statistical tests were two-sided and conducted using SAS 9.1 software.

References and Notes

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Acknowledgments: We thank N. Mall, J. Harding, and R. Basu for tissue sectioning and H&E staining; Y. Zhou for assistance with bioluminescence scanning; E. Fine, L. Jelicks, and W. Koba for help with microPET scanning at the Gruss Magnetic Resonance Research Center at Einstein; W. Guo for comments on the manuscript; and H. Ostrer for advice on human samples. Supported by U.S. Department of Defense Idea Development award W81XWH-07-1-0165 and NIH grants DK056638, HL069438, and HL097819. C.M. was the recipient of a fellowship from the Fondation pour la Recherche Médicale, France. Albert Einstein College of Medicine and the authors (C.M., P.S.F.) have filed a patent application (WO 2012071573 A2) relating to the use of adrenergic and muscarinic receptor antagonists for cancer therapy.

Supplementary Materials

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Figs. S1 to S12
Tables S1 to S5

11 February 2013; accepted 23 May 2013
10.1126/science.1236361

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Search for the Exit: Voyager 1 at Heliosphere's Border with the Galaxy

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We report measurements of energetic (>40 kiloelectron volts) charged particles on Voyager 1 from the interface region between the heliosheath, dominated by heated solar plasma, and the local interstellar medium, which is expected to contain cold nonsolar plasma and the galactic magnetic field. Particles of solar origin at Voyager 1, located at 18.5 billion kilometers (123 astronomical units) from the Sun, decreased by a factor of $>10^3$ on 25 August 2012, while those of galactic origin (cosmic rays) increased by 9.3% at the same time. Intensity changes appeared first for particles moving in the azimuthal direction and were followed by those moving in the radial and antiradial directions with respect to the solar radius vector. This unexpected heliospheric “depletion region” may form part of the interface between solar plasma and the galaxy.

Humankind's quest for exploration of the neighborhood of our solar system is currently embodied in the Voyager 1 and 2 (V1 and V2) spacecraft launched more than 35 years ago. V1 and V2 are currently at distances of 123 and 101 AU (1 AU = 1.5×10^8 km) from the Sun, at heliographic latitudes of 34.5° and -30.2° , respectively. Ideas about the dimensions and shape of the bubble of plasma called the heliosphere, created by the continuously flowing solar wind as the Sun travels through the local interstellar medium (LISM), are older than the space age (1). We present data from V1 showing that the intensities of energetic particles populating the hot heliosheath—the region between the solar wind termination shock [TS (2)] and the expected outer boundary, the heliopause—have suddenly decreased to instrumental background while galactic cosmic rays (GCRs) have simultaneously increased to levels thought to be characteristic of the LISM (3).

The V1 data used herein are from the Low Energy Charged Particle (LECP) instrument that measures differential intensities of ions with energies of 40 keV to ~ 60 MeV nuc^{-1} and of electrons with energies of 26 keV to >10 MeV (together with an integral ion measurement >211 MeV), determines the composition of ions with energies of >200 keV nuc^{-1} , and provides angular information via a mechanically stepped platform (4). Ion angular data provide estimates of plasma flow velocities at V1 (2) (fig. S1) in the absence of direct measurements from the V1 Plasma Science instrument (which failed in 1980). Data from other instruments on V1 are discussed in (5, 6).

The heliopause is expected to be a tangential discontinuity that separates the solar wind plasma

from plasma in the LISM. Models of the interface take into account the solar wind and LISM plasma, neutral (hydrogen atom) components, the interstellar and heliospheric magnetic fields, GCRs, anomalous cosmic rays (ACRs), and latitudinal and solar-cycle variations of the solar wind [(7, 8) and references therein]. V1 and V2 are the only space missions able to provide quantitative in situ measurements that can test the predictions of the various models.

LECP observations since 2004 have delineated the properties of the TS (2, 9) and have

detected the cessation of radial expansion of the solar wind within the heliosheath at ~ 113 AU (10), marking the beginning of a transition region while establishing that there was no statistically significant meridional flow at distances of >119 AU (11) at V1. The latter two observations have been interpreted differently in different models (12–14). Plasma flow has been mostly azimuthal, averaging ~ 26 km s^{-1} (11)—that is, in the direction opposite that of planetary motion. Subsequent LECP measurements on V1 over the past several months have revealed unexpected spatial and temporal structures, as discussed below.

Figure 1A shows changes of several percent in GCR intensity of both short (a few days) and long (a few months) duration in hourly averaged intensities since mid-2012. The overall increase from 2012.365 (7 May) to the beginning of 2013 was $\sim 30\%$. If the structures are stationary, V1 traveling at ~ 0.01 AU day^{-1} traverses 0.05 AU in 5 days; this is comparable to the gyroradius of a 1-GeV GCR (0.1 AU) in a magnetic field of 0.4 nT (6) and may be interpreted as spatial variations. The data in Fig. 1B demonstrate, however, that near-simultaneous decreases of an order of magnitude occurred within a single day in low-energy ions with gyroradii of only $\sim 3 \times 10^{-4}$ AU, a distance comparable to that traversed by V1 in an hour. Electrons with a gyroradius of $\sim 10^{-5}$ AU correspond to a V1 travel time of only a few minutes. We note, however, that electron intensities began a slow decay months earlier (at the

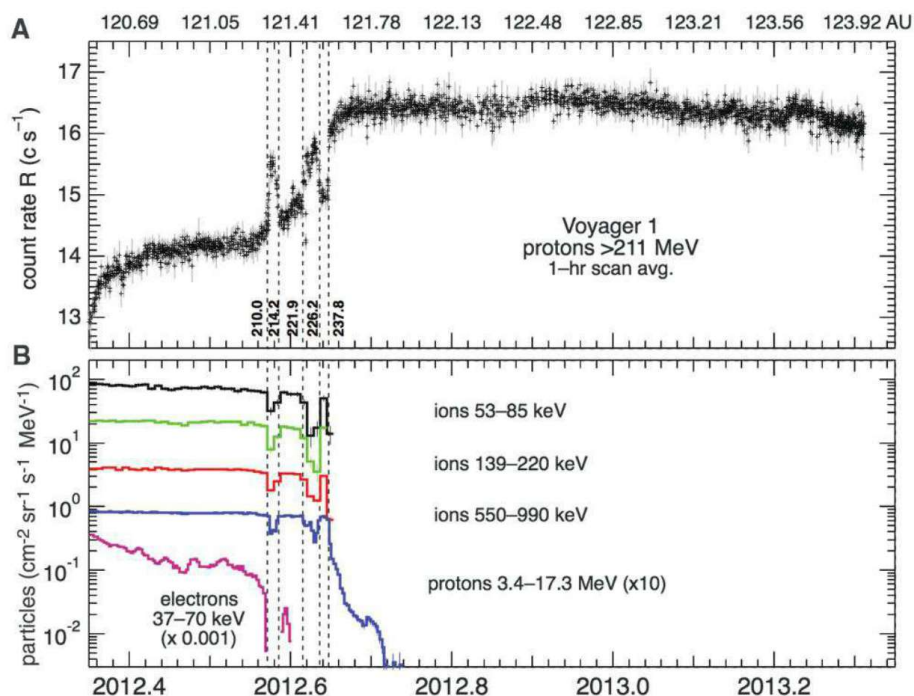


Fig. 1. Overview of energetic particle observations at V1, 2012.35 to 2013.40, showing the contrary behavior of GCRs and lower-energy particles. (A) Hourly averages of GCR activity and the pronounced boundary crossing on 25 August 2012 (day 238). GCR error bars are $\pm 1\sigma$. **(B)** Intensities of low- to medium-energy ions and low-energy electrons. The time evolution is very different, depending on energy and species.

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first onset of an increase in GCRs on 7 May), dropped precipitously on day 210 together with the ions, recovered briefly, but then disappeared before the second partial decrease on day 222. We address the nature of the sharp “edge” to the hot heliosheath on day 238 and its relationship

to the two “precursor” features by examining the intensity anisotropies measured by LECP.

Figure 2A shows that the two partial depletions of ~ 4 -MeV protons depended on the direction of motion of the particles with respect to the local magnetic field. As shown in the pie plots,

particles gyrating perpendicular to the magnetic field were depleted least, whereas those moving parallel to the field were depleted most, with a deeper depletion on day 231 than on day 212. During the subsequent flux recovery between day 233 and day 236, the fluxes were still anisotropic but with a clear-cut one-sided loss cone distribution, indicating that protons were streaming as previously (11) in the azimuthal ($-T$) direction (sector 3) but were now only weakly returning in the $+T$ direction along field lines beyond the spacecraft. The fluxes in the final ion decrease on day 238 were highly anisotropic, with the intensity of protons streaming along the $-T$ direction dropping first (sector 3), whereas those gyrating perpendicular to the magnetic field (sectors 1 and 5) decreased much more slowly. This “mirroring” distribution was maintained for more than 50 days, until 2012.71.

Figure 2B establishes that, despite the bidirectional response of our >211 -MeV proton channel, there must be anisotropies in GCRs throughout most of the period plotted. These anisotropies are particularly strong after the final GCR increase on day 238. Inspection of the pie plots shows that GCRs were roughly isotropic in mid-2012 but became gradually anisotropic during the brief spikes in intensity, and durably so after the final increase on 2012.63 (day 238). GCRs returned to near-isotropy on 2012.88, but the anisotropy reappeared on 2012.96 and increased thereafter. The presence of any anisotropy in GCRs, other than the well-known but small Compton-Getting anisotropy (15) observed when V1 was within the fast-flowing solar wind inside the TS, is in itself an unexpected finding. Long-held views on GCR acceleration and transport (16) incorporate steady isotropy in the LISM as a basic assumption, so the observed (time-dependent) GCR anisotropy implies that V1 is still in a region of transition to the LISM.

We note a clear transient increase in the >211 -MeV proton intensity during the period ~ 2013.22 to 2013.26 (days 080 to 095) that is apparent in both Figs. 1A and 2B. We suggest that this disturbance in the GCR intensities at 123.6 AU might be associated with a large global merged interaction region (GMIR) generated by the extraordinary solar activity beginning 5 March 2012 that contained three X-class and 19 M-class x-ray flares during the month from active regions near 15° to 20° N heliolatitude (V1 is at 34° N). The average velocity of the GMIR would have had to be ~ 120 AU $\text{year}^{-1} = 570$ km s^{-1} at some location along the field lines containing the GCRs sampled by V1 (but not necessarily at the spacecraft itself, because a nonlocal enhancement of the GCRs would propagate rapidly along the field lines).

To quantify the depletion of heliospheric particles and the enhancement of GCRs, we examined the energy spectra of the particle populations (H, He, C, and O) before and after the step like increases or decreases at the “edge” on day 238 (Fig. 3). We selected the 52-day period 2012.43 to 2012.57, before the first short-term depletion, as characteristic of the spectrum prior to the onset of

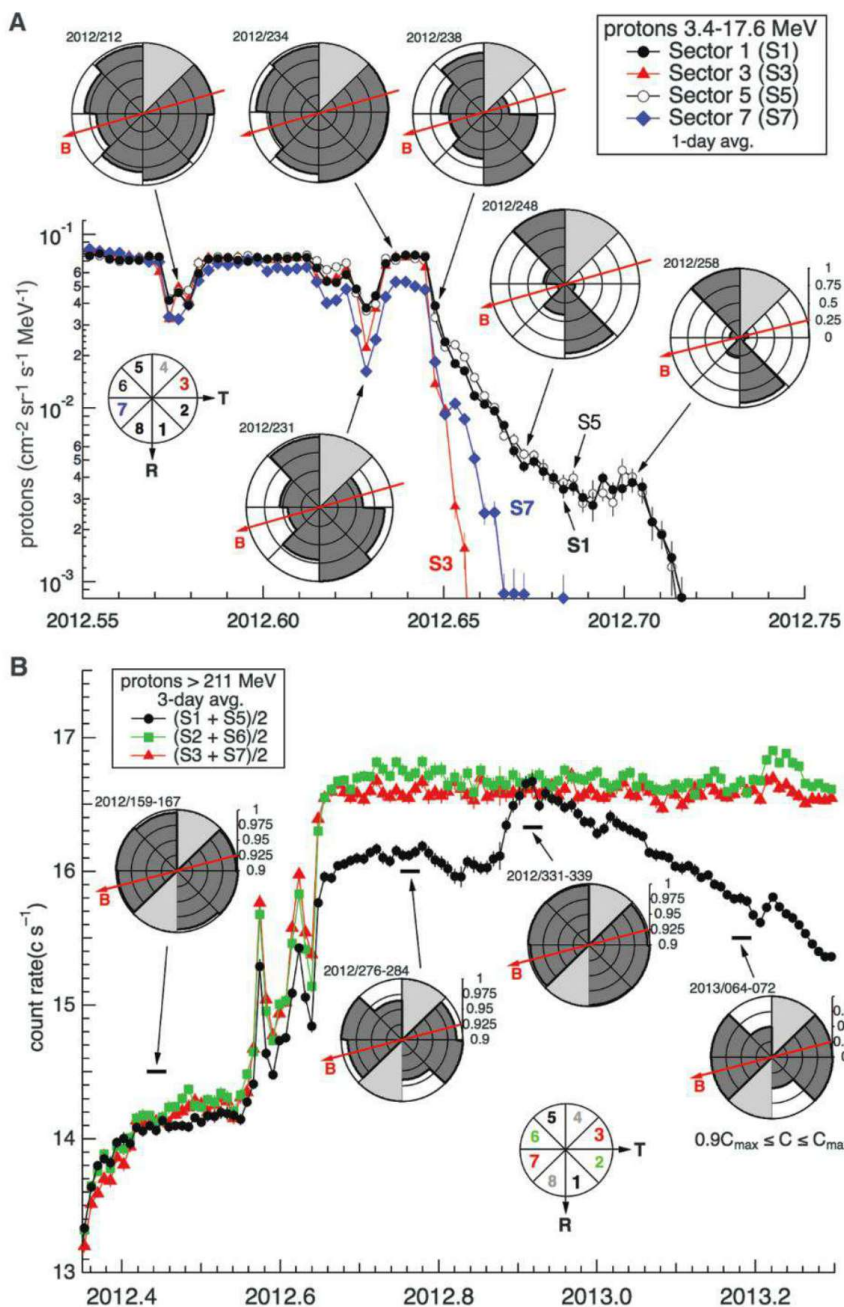


Fig. 2. Anisotropy measurements in ACRs and GCRs. (A) Time evolution of the anisotropy before, during, and after the boundary crossing. The pie plots represent angular distributions in the LECP scan plane, illustrated by the pinwheel diagram. The lightly shaded sector 4 is shielded (4), so measurements at low energies are not available. In the RTN coordinate system, R is the radius vector from the Sun, T is the cross-product of the solar rotation vector with R, and N completes a right-handed system. The red vector at each pie plot represents the direction of the magnetic field as measured by the onboard magnetometer (6). **(B)** Evidence of the anisotropy in GCRs revealed by the LECP measurements, using the bidirectional telescope (4). The lightly shaded sectors 4 and 8 are not plotted at present because of spacecraft mechanical obscuration (4). The pie plots reveal a difference between intensities parallel (azimuthal) and transverse (gyrating) to the measured magnetic field, especially after V1 crossed the “edge” on 2012.64.

the abrupt changes in intensities. The “after” period (2012.86 to 2013.0) begins following the ~ 4 -MeV proton return to relative isotropy on 2012.72 shown in Fig. 2A. The populations of H, He, and O contain particles of both heliospheric origin and ACRs at energies of <50 MeV nuc^{-1} . By contrast, C is a minor ACR component but is comparably abundant to O in GCR at energies of ≥ 20 MeV nuc^{-1} .

The “before” spectra are power laws in energy E of the form $\sim E^{-1.5}$ and steepen at higher energies, as were measured previously for many years (2, 16) and as were predicted (17). The “after” spectra are remarkably flat over this energy range for the three major ACR species H, He, and O. The flat O spectrum resembles that observed at similar intensities by the Ulysses spacecraft at high ($>55^\circ$) ecliptic latitudes (18) at 1.7 and 3.4 AU, which suggests that LISM field lines have a direct connection to the high-latitude heliosphere allowing GCR O direct access, much like solar particles have access to Earth’s polar caps. At lower (≤ 1 MeV nuc^{-1}) energies, there is a near-complete absence of fluxes, with upper limits shown (Fig. 3) for H, He, and O; all values are lower than the “before” intensities by factors of $>4 \times 10^3$ for H, He, and O and by a factor of >30 for C. At higher energies, H and He are decreased by varying factors, and O is lower for $E \leq 20$ MeV nuc^{-1} but unchanged above that energy. The C spectrum shows a small increase at $E \geq 20$ MeV nuc^{-1} , as expected from the primary GCRs.

The LECP observations presented here, taken by themselves, present a compelling case that V1 has crossed into a region of space that could be labeled “heliospheric depletion,” where hot heliosheath particles are undetectable at energies of >40 keV. If the predicted location of the putative heliopause at 121 AU deduced by combining energetic neutral atom observations and V1 in situ spectra (10) is interpreted as the hot plasma edge, it extends the depletion down to energies of ~ 5 keV. This view is supported by the observation that the increase in magnetic field pressure (6) by a factor of ~ 8 during the two partial depletions was accompanied by a decrease of only a factor of ~ 2 in the hot particle (>40 keV) pressure (Fig. 1), which suggests that most of the plasma pressure resides at energies of <40 keV.

In this region, ACRs, thought to be accelerated in the termination shock and/or its vicinity (19–21), are effectively absent, whereas GCRs have ready access and have increased to the highest level ever observed by LECP, presumably representative of intensities (3, 22) not modulated by heliospheric magnetic fields. The “emptying” of the hot heliosheath ions deduced from the LECP angular distributions began with magnetic (6) field-aligned flows in the azimuthal direction. This characteristic of a loss-cone distribution is observed for particles quasi-trapped in planetary magnetic fields but with little return flow at small pitch angles, implying an incomplete mirroring configuration. Particles gyrating perpendicular to the magnetic field also escaped the hot heliosheath, but at a slower rate. GCRs, on the other hand, flowed into the heliosheath preferentially

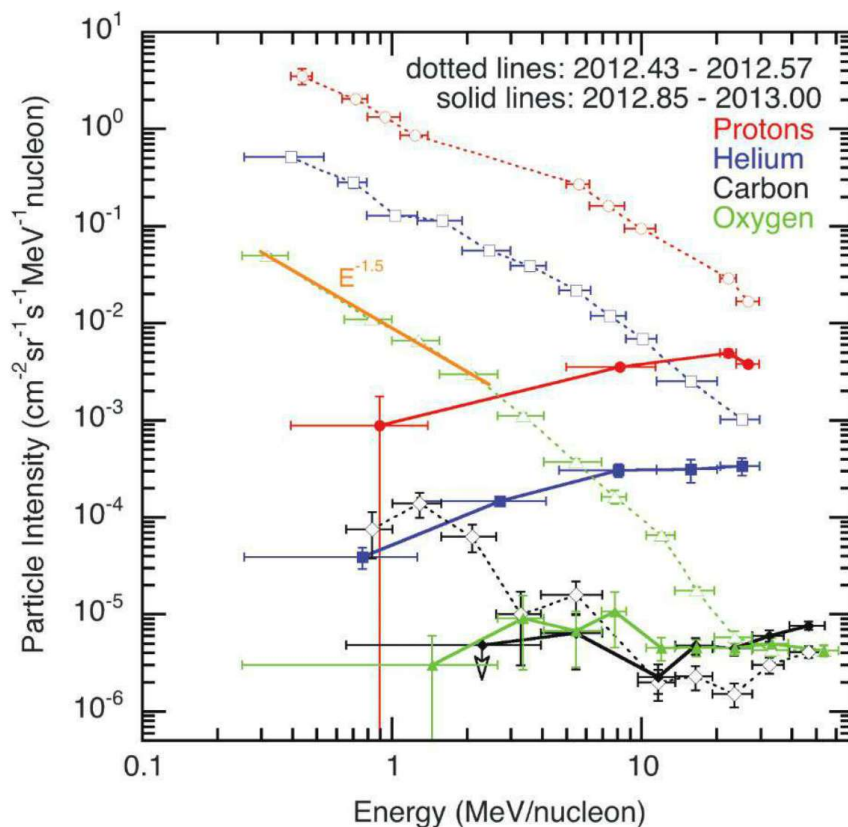


Fig. 3. Spectral evolution across the boundary. Fifty-two-day averaged energy spectra of heliosheath ions and lower-energy ACRs before (dashed lines, open symbols) and after (solid lines, solid symbols) the boundary crossing on 2012.64. Vertical error bars are statistical errors (often smaller than the symbol); horizontal bars indicate the energy band over which the fluxes are determined. The spectra of H, He, C, and O (red circles, blue squares, green triangles, and black diamonds, respectively) outside the boundary are remarkably flat in the LECP energy range and are remarkably consistent with high-latitude measurements from Ulysses in 1994 (18). See text.

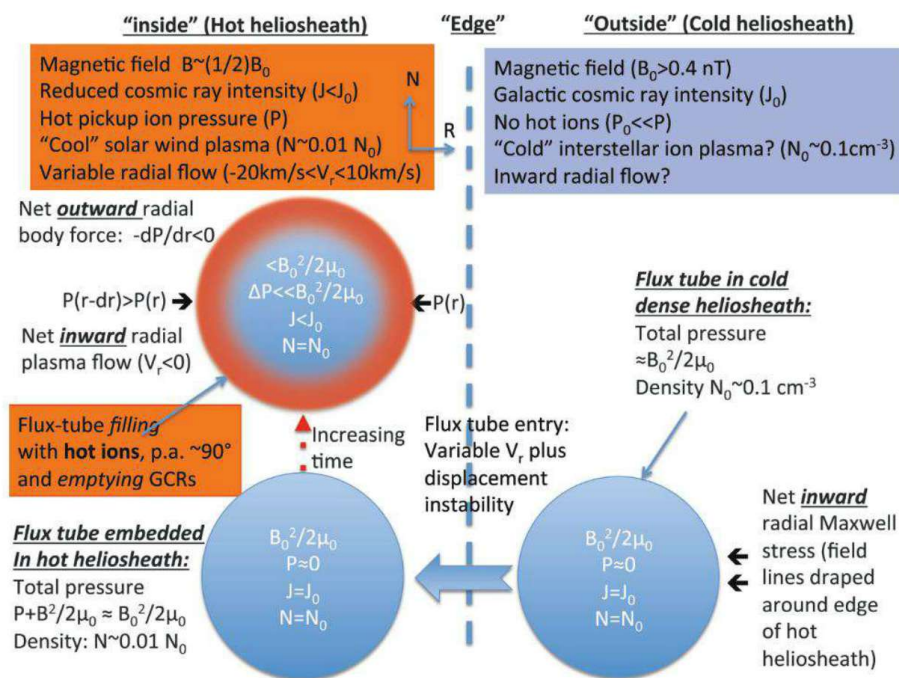
along the magnetic field, increasing to the highest intensity ever seen by LECP beyond the edge.

The main question is how the vicinity of the edge encountered by V1 differs from characteristics expected for the LISM. Some models have suggested an increase in magnetic field (23); others have predicted only a gradual drop in ACRs and a gradual increase in GCRs (24). No model predicted either the extreme sharpness of the edge (in all particle species and the magnetic field) or the discontinuities in these quantities preceding the edge crossing. The invariance of the magnetic field direction across the edge during the crossing on day 238 (6) was totally unexpected. In Fig. 4, we suggest plasma processes (25) in the vicinity of the edge that are consistent with these observations. A key element is that for the year preceding the crossing (2011.6 to 2012.6), the average radial plasma flow V_r in the heliosheath fluctuated between positive and negative values (fig. S1) (11, 26) but was mostly negative ($-20 \text{ km s}^{-1} < V_r < +10 \text{ km s}^{-1}$), as determined from anisotropies of >40 -keV ions. The curvature of the boundary is concave inward, so the Maxwell stresses tend to pull flux tubes just outside the edge into the heliosheath. But the hot ion pressure inside the heliosheath is decreasing toward the edge, cre-

ating an outward body force on the plasma that resists the entry of the cold flux tubes.

Consequently, the system may be susceptible to an interchange instability (25), thus allowing the cold, high-magnetic field flux tubes to enter the heliosheath. The most favorable situation for instability is if the tangential direction of the magnetic field is the same on both sides of the boundary, in agreement with the magnetic field observations (6). However, the effect of flows across the boundary are not usually considered in the classical interchange instabilities. For flux tubes already inside the heliosheath but near the edge, the mainly negative radial flow will tend to keep them from crossing the edge. Because of the same mainly negative fluctuations, a LISM flux tube with strong magnetic field and containing only GCRs and cold interstellar plasma that becomes embedded into the edge of the hot heliosheath by the instability will tend to move deeper within the heliosheath. It will then begin to be populated with hot ions, first at 90° pitch angles (day 238) and then at all other angles (day 234) (Fig. 2A). At the same time, the GCRs will slowly leak out the flux tube into the heliosheath, thus decreasing their intensity (Fig. 2B). All the while, the magnetic field intensity will keep most of its

Fig. 4. Schematic summary of salient observations and their possible explanation near the “edge.” The illustration is drawn in the heliographic R-N plane, so the measured magnetic field direction (which does not change across the edge) is approximately perpendicular to the page. The “hot heliosheath” (HH) is in pressure balance (P) from hot ions plus magnetic field ($B^2/2\mu_0$, where $\mu_0 = 4\pi \times 10^{-7}$) with the “cold heliosheath” (CH) with plasma density $N_0 > N$. Beyond the edge, the CH is dominated by magnetic pressure ($B_0^2/2\mu_0$) and is devoid of hot ions but contains higher intensities (J_0) of GCRs. We expect the “cold dense” plasma density N_0 in the CH to be near the interstellar value. The inward radial Maxwell stress (due to draping of CH field lines around the HH) and the inward particle pressure gradient in the HH (because of the reduction in hot ion intensities) suggest the possibility of a displacement instability at the edge that could allow flux tubes from the CH to invade the HH, where they would commence filling with hot ions while losing their GCRs ($J < J_0$). The fluctuating radial flows ($-20 \text{ km s}^{-1} < V_r < 10 \text{ km s}^{-1}$) are negative (on average) in the HH (11) (fig. S1), so this will tend to draw invading flux tubes deeper into the HH while suppressing the crossing of HH flux tubes across the edge.



outside value (B_0) in order to maintain pressure balance. The two “precursors” before the edge thus may be the signatures of such invasive flux tubes from beyond the edge. We then suggest that the reason for the location (and the sharpness) of the edge itself is that the conditions favoring this modified flux tube interchange process must be very sensitive to the ratio of the hot ion pressure (P , decreasing outward) and the magnetic pressure ($B^2/2\mu_0$, increasing outward), while their sum is constrained by pressure balance to the value $B_0^2/2\mu_0$ (magnetic pressure beyond the edge).

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Acknowledgments: Work at the Johns Hopkins University Applied Physics Laboratory is supported by NASA contract NNN06AA01C and by subcontracts at the University of Maryland and Fundamental Technologies LLC. We thank J. Gunther, L. Brown, and S. Lasley for assistance in the data analyses efforts.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1235721/DC1 Fig. S1

28 January 2013; accepted 4 June 2013
Published online 27 June 2013;
10.1126/science.1235721

Magnetic Field Observations as Voyager 1 Entered the Heliosheath Depletion Region

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Magnetic fields measured by Voyager 1 (V1) show that the spacecraft crossed the boundary of an unexpected region five times between days 210 and ~238 in 2012. The magnetic field strength B increased across this boundary from ≈ 0.2 to ≈ 0.4 nanotesla, and B remained near 0.4 nanotesla until at least day 270, 2012. The strong magnetic fields were associated with unusually low counting rates of >0.5 mega-electron volt per nuclear particle. The direction of $\mathbf{B}$ did not change significantly across any of the five boundary crossings; it was very uniform and very close to the spiral magnetic field direction, which was observed throughout the heliosheath. The observations indicate that V1 entered a region of the heliosheath (the heliosheath depletion region), rather than the interstellar medium.

Voyager 1 (V1) crossed the termination shock and entered the heliosheath on ≈ 16 December 2004, moving in the north-

ern hemisphere $\sim 34.5^\circ$ above the solar equatorial plane in the general direction of the nose of the heliosphere. V1 has moved radially from 94

to 121 astronomical units (AU) at 34.5°N in the heliosheath since it crossed the termination shock (1–3). Recent estimates of the position of the heliopause (the boundary of the heliosheath and the interstellar medium) along the V1 trajectory range from ~110 to 150 AU (4–6). During 2010, the radial component of the velocity at the position of V1 was near 0 km/s (7), and from day of the year (DOY) 126, 2010 through 308, 2011, the average northward component of the velocity was 28 ± 3 km/s (8) (DOY 1 = 1 January). The speed slowed during 2011 to form a quasi-stagnation region extending from 113 to beyond 119 AU (8, 9), suggesting that V1 may be approaching the heliopause.

We present V1 magnetic field observations from 150, 2012 through 270, 2012 in Fig. 1. The particles >0.5 mega-electron volt per nuclear particle (MeV/nuc) are discussed in more detail in (10, 11), and the magnetometer and data are described in (12, 13) and in the supplementary materials. During this interval, V1 was at 34.5°N, moving from 120.7 to 121.9 AU radially away from the Sun. From 150, 2000 to 210, 2012, there is no correlation between the magnetic field strength B (which varies from 0.072 to 0.36 nT) and the counting rate of particles >0.5 MeV/nuc (14) (which remains nearly constant). In contrast, from 210, 2012 to 270, 2012 there is a strong anticorrelation between B and the particle counting rates.

Figure 1 shows a series of jumps in B starting on 210, 2012 and ending on 240, 2012, labeled B1 to B5 (Table 1). The jumps indicate multiple crossings of a boundary unlike anything observed previously by V1. On 210, 2012 (B1), B increased abruptly from 0.17 to 0.43 nT (the strongest magnetic fields observed by V1 in the heliosheath since crossing the termination shock in 2004), and the particle counting rate dropped by a factor of ~2 (from 23.6 to 12.2 counts/s) at the same time. The energetic particle data show that jump B3 did not correspond to a complete entry into the region beyond the boundary, because the counting rates did not drop to near

the minimum value and B did not rise to the level observed after B1 and B5. At the last jump on ~238, 2012 (B5), B increased to ~0.43 nT and it remained at that value until at least 270, 2012, while the particle counting rate dropped from ~25 counts/s to ~2 counts/s (background) until at least 270, 2012.

In the heliosheath, the average magnetic B between the termination shock crossing at the end of 2004 and the beginning of 2011 was 0.1 nT, corresponding to a magnetic pressure $B^2/8\pi = 0.04 \times 10^{-12}$ dyn cm⁻² = 0.004 pPa. In the heliosheath depletion region (HDR), B is 0.44 ± 0.01 nT and the magnetic pressure is $B^2/8\pi = 0.8 \times 10^{-12}$ dyn cm⁻² = 0.08 pPa, nearly 20 times greater than observed for 5 years after the termination shock crossing.

The enhancements of B between B1 and B2, between B3 and B4, and after B5 are possibly largely the response of B to the decrease in pressure caused by loss of the energetic particles, in order to maintain pressure balance normal to B and equilibrium in the region. In this case, the

boundaries are pressure-balanced structures (15), which correspond to MHD tangential discontinuities such as stream interfaces.

It is generally assumed that the heliopause is a pressure-balanced structure (or tangential discontinuity in the MHD approximation), which is possibly rippled by waves and turbulence generated by instabilities (16, 17) and punctuated by reconnection events (6). Because the observations above suggest that the boundaries observed by V1 during 2012 are pressure-balanced structures, one must consider the hypothesis that the boundaries represent multiple crossings of the heliopause and that V1 has entered the interstellar medium.

Because of the rotation of the Sun, the solar magnetic field forms the Parker spiral field as it is carried radially outward by the solar wind (18), which is observed to have an east-west orientation at the position of V1. In contrast, the 10-AU difference in the location of the termination shock in the northern and southern hemispheres implies that the interstellar magnetic

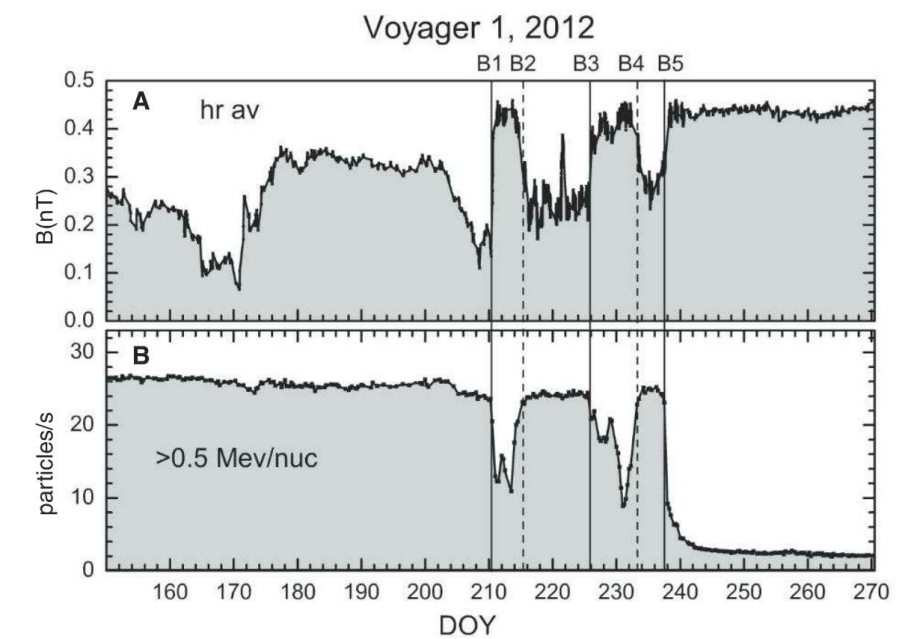


Fig. 1. Relationship between the magnetic field intensity and the energetic particle counting rate. Hour averages of magnetic field strength B (A). The counting rate of energetic particles >0.5 MeV/nuc to ~30 MeV (B).

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Table 1. Changes in B at the boundaries of the HDR.

t_o (days)	Angles				Angle changes		Parameters for $B(t)$			
	λ_L°	λ_H°	δ_L°	δ_H°	$ \lambda_H - \lambda_L ^\circ$	$ \delta_H - \delta_L ^\circ$	B_L (nT)	B_H (nT)	w (hours)	
B1	210.6	275 ± 7	282 ± 1	5 ± 9	12 ± 1	7 ± 7	7 ± 9	0.170	0.425	5.3
B2	215.6	295 ± 11	282 ± 2	19 ± 5	11 ± 2	13 ± 11	8 ± 5	0.236	0.416	8.6
B3	225.7	285 ± 3	285 ± 2	13 ± 3	15 ± 3	0 ± 4	2 ± 4	(0.249)	(0.372)	<10.7
B4	233.5	284 ± 4	286 ± 1	13 ± 3	17 ± 2	2 ± 4	4 ± 4	0.271	0.425	35.4
B5	237.7	286 ± 3	287 ± 1	12 ± 2	12 ± 1	1 ± 3	0 ± 2	0.272	0.438	18.4
Average		285.8 ± 1.8	284.8 ± 0.5	12.8 ± 1.4	12.5 ± 0.6	1.8 ± 1.9	1.8 ± 1.5	0.237	0.426	11.9

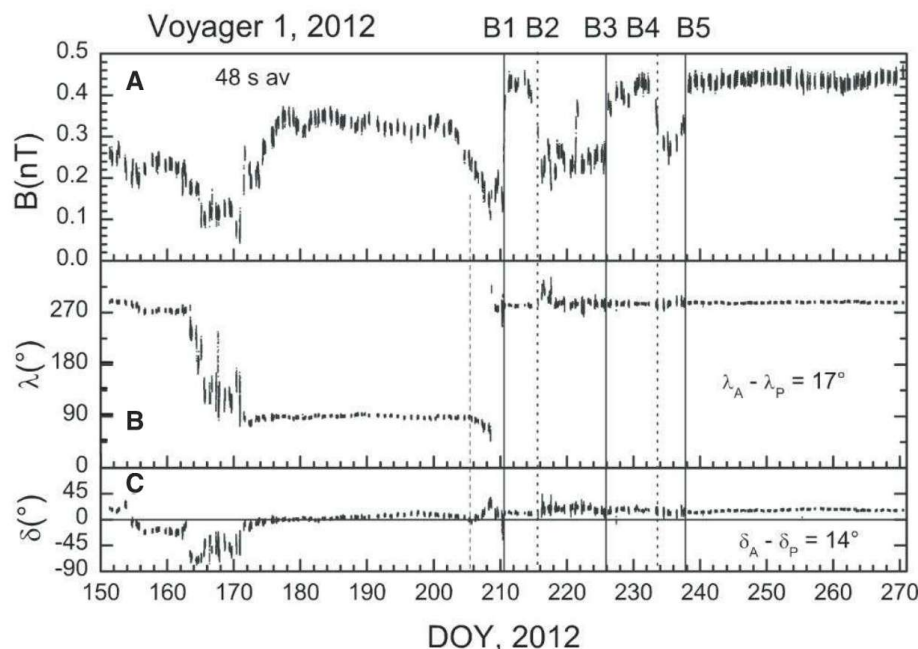


Fig. 2. High-resolution observations of the magnetic field strength and direction. 48-s averages of the magnetic field strength B (A), azimuthal angle λ (B), and elevation angle δ (C), as a function of time measured from DOY 150 to 270, 2012. The angles are in RTN coordinates (28). Before 210, 2012, V1 observed magnetic fields characteristic of the heliosheath (26). The elevation and azimuthal angles are close to the Parker spiral direction, $\delta_P \approx 0^\circ$ and $\lambda_P \approx 90^\circ$ or 270° , respectively. A magnetic sector in which B was directed sunward along the Parker spiral angle was observed between 171, 2012 and 208, 2012. The magnetic field strength varied from 0.07 to 3.36 nT before the boundary crossings.

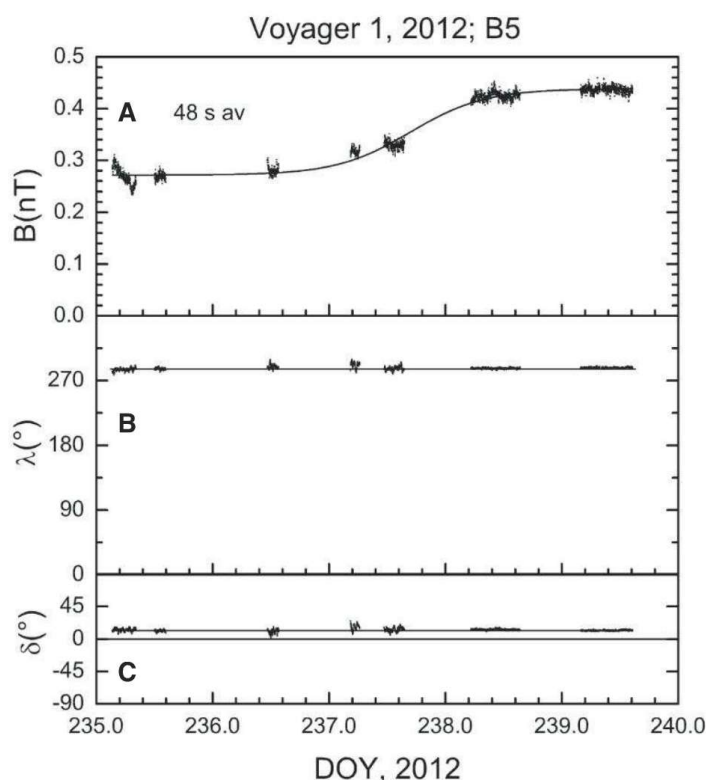


Fig. 3. The magnetic profile as V1 passed through the boundary into the HDR. (A to C) 48-s averages of the magnetic field profile during the fifth crossing of the boundary into the heliosheath depletion region (Fig. 2). The solid curve is a sigmoid function, $B(t) = B_2 + [B_1 - B_2]/[1 + \exp(t - t_0)/(w/4.4)]$, which provides an excellent fit to the data (coefficient of determination $R^2 = 0.98$). The parameter w gives the time required for B to change from 10 to 90% of the way to the asymptotic values (15). t is the time in DOY; t_0 is a parameter that corresponds to the center of the profile.

field must have a component in the north-south direction (19–21) and is not parallel to the east-west direction of the solar magnetic field in the heliosheath. Consequently, the magnetic field direction should change when V1 crosses the heliopause (fig. S1). The magnetic field direction could remain constant across the heliopause only if the interstellar magnetic field were nearly parallel to the solar ecliptic plane and tangential to the heliospheric magnetic field. Such a configuration is highly improbable and would have to be a remarkable coincidence, because the interstellar magnetic field has no causal relation to the solar magnetic field (22, 23).

Higher-resolution magnetic field observations from 210, 2012 to 270, 2012 (Fig. 2) suggest that V1 did not observe a significant change in the direction of B at any of the five crossings of the boundary. Table 1 shows the angles on the low field (subscript L) and high field (subscript H) sides of each boundary crossing as well as the absolute value of the differences of these angles. The changes in the direction of B for each of the five boundary crossings are indeed very small. The weighted averages of the changes in direction angles are $\langle \Delta \lambda \rangle = \langle \lambda_H - \lambda_L \rangle = 1.8^\circ \pm 1.9^\circ$ and $\langle \Delta \delta \rangle = \langle \delta_H - \delta_L \rangle = 1.8^\circ \pm 1.5^\circ$, consistent with no change in the direction of B .

During the last boundary crossing (Fig. 3), the strength of B increased from 0.272 to 0.438 nT during an interval of ≈ 18.4 hours centered at day 237.7. The changes in the angles across B5 are $\Delta \delta = 0^\circ \pm 2^\circ$ and $\Delta \lambda = 1^\circ \pm 3^\circ$. Because the uncertainties refer to differences of angles within one day, they probably represent statistical uncertainties, relatively unaffected by drifts and other systematic errors. Because there was no change in the direction of B with a high degree of certainty, it is very unlikely that the boundary B5 is the heliopause.

The magnetic properties of the HDR from 238, 2012 to at least 270, 2012 define the region, because they differ from all previous observations within the heliosheath. The average magnetic field strength is 0.436 ± 0.010 nT. An interstellar magnetic field strength of this magnitude or greater has been ruled out as being too high to explain the ribbon of energetic neutral particles discovered by NASA's Interstellar Boundary Explorer (24), which adds support to our conclusion that the HDR is associated with the heliosheath rather than the interstellar medium. The magnetic field vector in the RTN coordinate system is $B = (0.126 \pm 0.008, 0.400 \pm 0.010, 0.120 \pm 0.013)$ nT. The uncertainties in these average values are the standard deviations, and their values are close to the digitization level and root mean square noise of the instrument, 0.004 and 0.003 nT, respectively. Thus, the fluctuations in the components of B are extremely small in the HDR. The region is not turbulent.

The average direction of the magnetic field in the HDR is $\lambda_A = 287^\circ \pm 1^\circ$ and $\delta_A = 14^\circ \pm 2^\circ$. The average magnetic field direction is close to the Parker spiral magnetic field direction (Fig. 2),

but there is a statistically significant difference from the spiral field direction in the HDR, namely $\lambda_A - \lambda_P = 17^\circ \pm 1^\circ$ and $\delta_A - \delta_P = 14^\circ \pm 2^\circ$ as shown in Fig. 2. The magnetic polarity of the magnetic field in the HDR indicates that it has moved from the southern hemisphere to the position of V1 in the northern hemisphere. The small departure from the spiral field direction might be the result of a flow that carried the magnetic field northward in the heliosheath to the location of V1. It has been suggested that such a flow moves northward in the heliosheath between a “magnetic wall” or “magnetic barrier” and the heliopause at the latitude of V1 (5, 25).

Increasingly strong magnetic fields from the middle of 2010 until at least the middle of 2011 (possibly extending up to 150, 2012 as shown in this paper) were reported in (26), where it was suggested that these strong magnetic fields might be related to a magnetic wall or magnetic barrier. Thus, it is conceivable that the HDR corresponds to this northward heliosheath flow near the heliopause, and the boundary of the HDR represents a boundary of material that was moving radially closer to the Sun. The strong magnetic fields observed from mid-2010 to 270, 2012 could be an interaction region that extends into the HDR, produced by the collision of these two flows. The stronger magnetic field in the HDR might be produced in response to the reduction of pressure owing to the absence of energetic particles. The absence of energetic particles could indicate that magnetic lines passing V1 were no longer

connected to their source (the blunt termination shock), because V1 crossed a topologic boundary in the magnetic field of the inner heliosheath beyond the last magnetic connection point to the termination shock (27). Alternatively, the energetic particles could have escaped into interstellar space, if the heliosheath magnetic field reconnected with the interstellar magnetic field beyond the position of V1.

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Acknowledgments: T. McClanahan and S. Kramer provided support in the processing of the data and D. Berdichevsky computed correction tables for the three sensors on each of the two magnetometers. N.F.N. was partially supported by NASA grant NNX12AC63G to the Catholic University of America. L.F.B. was supported by NASA contract NNG11PN48P. The data are available at NASA's Virtual Heliospheric Observatory (<http://vho.nasa.gov/>), maintained within the Heliospheric Physics Laboratory at NASA's Goddard Space Flight Center.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1235451/DC1
Supplementary Text
Fig. S1

21 January 2013; accepted 30 May 2013
Published online 27 June 2013;
10.1126/science.1235451

Voyager 1 Observes Low-Energy Galactic Cosmic Rays in a Region Depleted of Heliospheric Ions

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On 25 August 2012, Voyager 1 was at 122 astronomical units when the steady intensity of low-energy ions it had observed for the previous 6 years suddenly dropped for a third time and soon completely disappeared as the ions streamed away into interstellar space. Although the magnetic field observations indicate that Voyager 1 remained inside the heliosphere, the intensity of cosmic ray nuclei from outside the heliosphere abruptly increased. We report the spectra of galactic cosmic rays down to $\sim 3 \times 10^6$ electron volts per nucleon, revealing H and He energy spectra with broad peaks from 10×10^6 to 40×10^6 electron volts per nucleon and an increasing galactic cosmic-ray electron intensity down to $\sim 10 \times 10^6$ electron volts.

A key objective of the Voyager Cosmic Ray Subsystem (1) is the determination of the intensity of galactic cosmic-ray

(GCR) nuclei and electrons in the interstellar medium outside of the heliosphere. On 25 August 2012, Voyager 1 (V1) entered a region where the heliospheric ions were depleted and replaced by low-energy GCR nuclei and electrons. This would have been expected had V1 crossed the heliopause, the boundary separating the solar wind plasma and magnetic field from the interstellar plasma and magnetic field. However, there was no change in the direction of the

magnetic field even though the field intensity abruptly increased by 60%, indicating that the magnetic field lines in this region originated at the Sun, not from interstellar space (2). So, V1 appears to have entered a previously unknown region that is depleted of energetic heliospheric ions and accessible to low-energy cosmic rays [see also (3, 4)].

The first indication of a heliospheric depletion region was observed on 28 July 2012, when the intensity of protons from inside the heliosphere with energies $0.5 \text{ MeV} \leq E \leq 60 \text{ MeV}$ abruptly decreased and subsequently recovered 5 days later (counting rates C and D in Fig. 1). A second decrease on 13 August lasted 8 days and was followed 4 days later by the durable entry of V1 into the heliospheric depletion region on 25 August. The magnetic field increased simultaneously with the decreases in energetic protons, suggesting that lower-energy plasma may also have escaped, with the resulting decrease in plasma pressure leading to a compression of the magnetic field (2).

The intensity changes for four distinct populations of energetic particles are strongly correlated as shown in Fig. 1. Because of their small mass, the GCR electrons have the smallest radii of gyration around the magnetic field lines, typically 0.0006 astronomical units (AU) for a

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10-MeV electron in a heliosheath magnetic field of 0.4 nT. Because V1 crosses that distance in <1.5 hours, the electrons provide the sharpest indication of when V1 crossed the boundary of the region where there is enhanced access of GCRs from outside. The edges of the regions of

enhanced electron intensities are closely aligned with the five boundaries of the regions of enhanced magnetic field that occurred on day of the year (DOY) 210.6, 215.6, 225.7, 233.5, and 237.7 (2). The heavier ions have larger gyroradii that result in broader intensity transitions, as

seen in the counting rate of >70-MeV cosmic-ray nuclei. For example, V1 crosses the 0.025-AU gyroradius of a 100-MeV proton in ~2.5 days. Anomalous cosmic rays (ACRs) are also accelerated in the outer heliosphere, and at energies below ~100 MeV per nucleon their intensity

Fig. 1. The counting rates (6-hour averages) of four different energetic particle species in the vicinity of the depletion region. (A) (y axis on right) GCR nuclei (mainly protons with $E > 70$ MeV) penetrating the High Energy Telescope 1 (HET 1). **(B)** (y axis on left) GCR electrons with energies between 6 and ~100 MeV observed by the Electron Telescope (TET). **(C)** (y axis on left) Protons with 7 to 60 MeV stopping in HET 1 (rate shown is divided by 11.55) are mainly anomalous cosmic rays before 2012/238 (25 August) and galactic cosmic rays after that. **(D)** (y axis on left) Low-energy particles observed in the LET A (rate shown is divided by 124.5) are mainly protons with 0.5 to ~30 MeV accelerated at the termination shock and in the heliosheath plus a scaled background rate of 0.017 s^{-1} because of higher-energy nuclei. Three distinct periods in 2012 on days 210 to 215 (28 July to 2 August), 226 to 233 (13 to 20 August), and from 238 (25 August) are indicated by vertical lines corresponding to the magnetic boundaries of the depletion region (2). The simultaneous intensity changes coincide with abrupt increases and decreases in the magnitude of the magnetic field, suggesting that, after two brief encounters with a depletion region or regions, V1 durably entered a broad depletion region on 25 August (DOY 238).

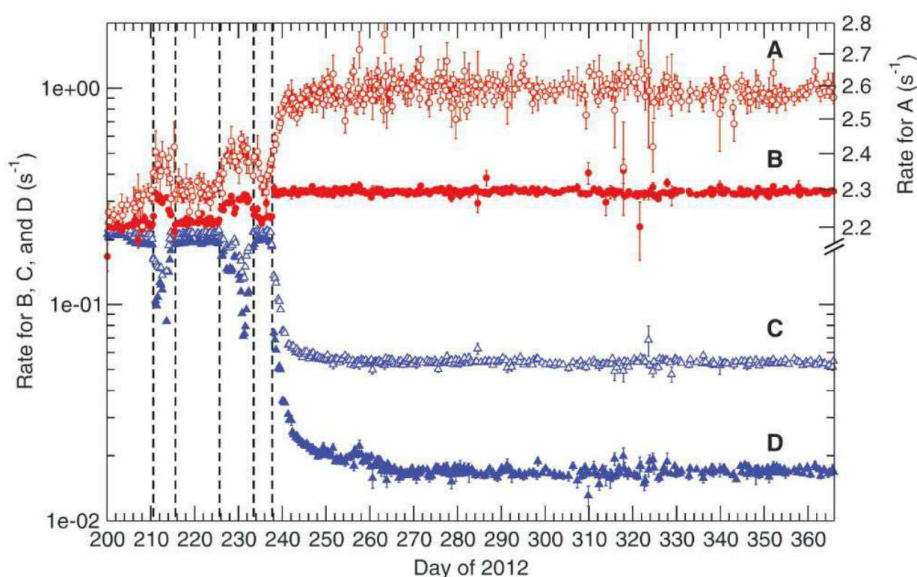
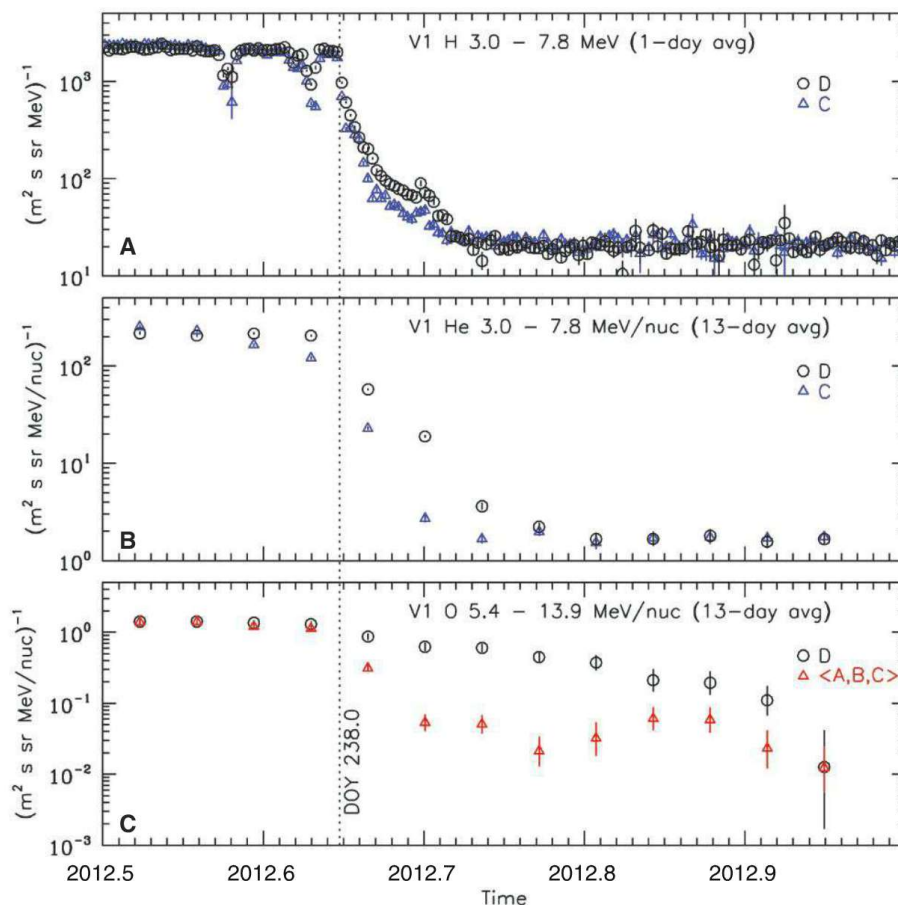


Fig. 2. Intensities of H, He, and O from V1 for the last half of 2012. (A) One-day average intensities of H with 3.0 to 7.8 MeV. Intensities are shown for two of the four LETs [see (1) for arrangement of the telescopes]. The bore sight of LET D is pointed roughly perpendicular to the magnetic field direction. The bore sight of LET C is oriented at 90° to that of LET D. **(B)** Similar to (A) except for 13-day averages of the intensities of He with 3.0 to 7.8 MeV per nucleon. **(C)** Similar to (B) except for O with 5.4 to 13.9 MeV per nucleon, and the average intensity from the LET A, B, and C is plotted instead of only LET C, in order to improve the statistical significance of the result. The LET A bore sight is oppositely directed to that of LET C, and the bore sights of LET A, B, and D form an orthogonal set. Error bars indicate statistical uncertainties ($\pm 1 \sigma$).



greatly exceeds that of GCRs. As seen by the intensity of 7- to 60-MeV protons in Fig. 1, the intensities of ACRs also decrease in the depletion regions as they escape out of the heliosphere. Their disappearance beginning on DOY 238 reveals the intensity of GCRs of the same energy that have flowed into the depletion region from outside.

Voyager has four Low-Energy Telescopes (LETs) arranged in an orthogonal array (1). As illustrated in Fig. 2, there are substantial differences in intensity among the telescopes over extended periods. LET D is oriented so that it observes protons with pitch angles from 50 to 100 to the spiral magnetic field, which is pointing outward along the spiral direction (2), so LET D is sensitive to ions moving outward along the field ($\theta < 90^\circ$) and also inward ($\theta > 90^\circ$). LET C observes protons with pitch angles of from 110 to 160, so it is sensitive to ions coming inward along the spiral field.

These ions originate at the termination shock or in the heliosheath and diffuse mainly along the spiral magnetic field. Before 28 July, there was sufficient scattering on the field line that the intensity of ions in LET C was the same as in LET D. However, during the first two decreases, the intensity of ions diffusing inward toward LET C was significantly lower than in LET D, indicating some of the ions spiraling outward were lost and not scattered back toward V1.

After the boundary crossing on 25 August (DOY 238), the intensity of H ions (protons) in LET C dropped much more rapidly than the intensity of protons in LET D. The two rates converged after 2012.72 (DOY 263), indicating that low-energy protons from the heliosphere were no longer dominating the intensity near 5 MeV at V1. Instead, the intensity was isotropic, as expected if the remaining protons are low-energy GCRs diffusing in along the magnetic field.

Figure 2 also shows similar anisotropies for He and O. Although longer time averages are required because of the lower intensities, there is evidence for losses in LET C during the events before DOY 238 and for extended periods after. At those times, the outward flow was observed in LET D, while LET C was already observing a lower intensity of isotropic GCRs diffusing inward along the magnetic field. The longer persistence of the heavier heliospheric ions is consistent with the expectation that singly ionized 5-MeV per nucleon He^+ and 9-MeV per nucleon O^+ with gyroradii of 0.022 and 0.12 AU, respectively, will have larger scattering mean free paths and will be scattered into the loss cone more slowly than 5-MeV H^+ , which has a gyroradius of 0.0054 AU.

The disappearance of most of the heliospheric ions after 25 August 2012 provides an opportunity to examine the energy spectra of GCRs to lower energies than previously possible (4). Figure 2 shows that GCR H and He dominate the 3- to 7.8-MeV per nucleon energy range

Fig. 3. Differential energy spectra of H, He, C, and O from V1.

Two spectra are shown for H: one for a reference period before the depletion region was reached, 2011/274 to 2012/121, and one for a new period within the depletion region, 2012/303 to 366. For these two H spectra, intensities from all four LETs were averaged together. At higher energies, >57 MeV, only intensities from HET 2 are shown. The same telescopes were used in deriving the He spectrum for the period 2012/303 to 366. For C, intensities from all four LETs were used, and at higher energies, >20 MeV per nucleon, intensities from both HETs were averaged together. For O with 5.4 to 17.1 MeV per nucleon, only LET A, B, and C were used in order to minimize the contribution from heliospheric particles. For O with 17.1 to 21.6 MeV per nucleon, only HET 2 was used, and for energies >21.6 MeV per nucleon, intensities from both HETs were averaged together. The C and O spectra are for the period 2012/261 to 366. Several estimates of the local interstellar galactic cosmic ray H and He spectra are shown. The solid lines are model a from Ip and Axford (13). The dotted lines represent the leaky-box model from Webber and Higbie (14). The dashed lines are the DC model from Moskalenko *et al.* (19), and the dot-dash line for H is from Fisk and Gloeckler (20). Error bars indicate statistical uncertainties ($\pm 1 \sigma$).

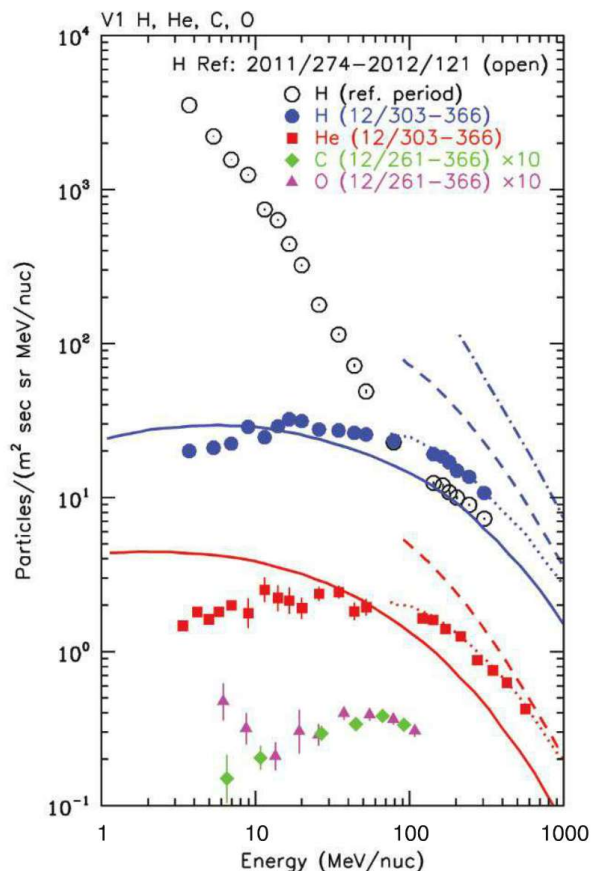
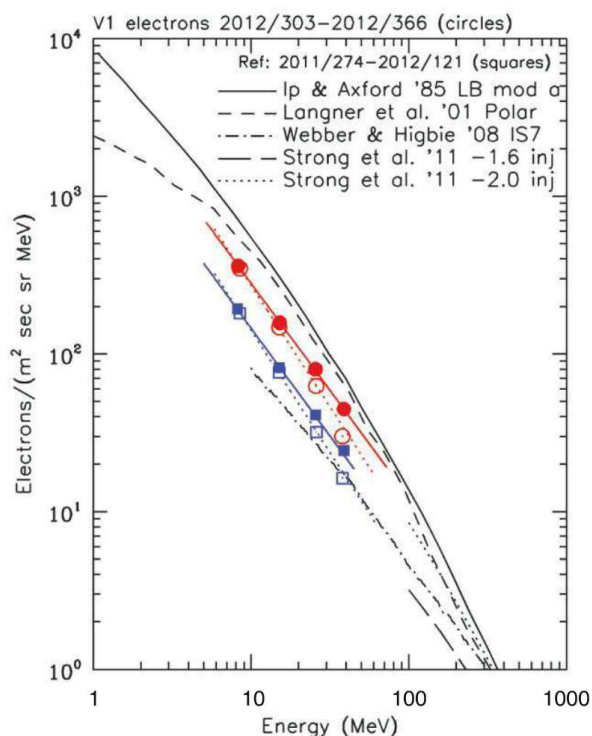


Fig. 4. Differential energy spectra of electrons from the V1 TET.

Two pairs of spectra are shown, one pair for a reference period before the new region was reached, 2011/274 to 2012/121, and one pair for a period within the new region, 2012/303 to 366. The open symbols represent spectra derived by using response functions from a prelaunch accelerator calibration. The solid symbols use response functions from a GEANT4 simulation. The intensity differences in the solid and open symbols for a given period are an indication of the systematic uncertainty in the electron spectrum that is proportional to $E^{-1.45 \pm 0.09}$ in the new region. The method used in deriving the energy spectra is described in the supplementary materials. Three estimates of the local interstellar GCR electron spectrum are shown. The solid line is model a from Ip and Axford (13), the dot-dash line represents model IS7 from Webber and Higbie (21), the short-dashed line represents the polar model from Langner *et al.* (16), and the long-dashed and dotted lines are from Strong *et al.* (17) for electron source spectra proportional to $E^{-1.6}$ and $E^{-2.0}$, respectively, and include positrons.



from ~2012.77 (DOY 282) onward, because the intensity apparently became isotropic at that time, consistent with the expectation for GCRs. Figure 3 shows the energy spectra for the period 2012/303 to 366 for H and He from 3 to several hundred MeV per nucleon along with an energy spectrum of H from a period before the onset of the recent activity. This reference spectrum shows the dominance of the spectrum by the termination shock particle and ACR heliospheric particle populations below ~100 MeV per nucleon during this time. These particles have largely streamed away in the more recent period (DOY 303 to 366), replaced by the inflow of low-energy GCRs.

However, it is uncertain whether GCRs have fully unimpeded access into this region. In addition, the GCR intensity immediately outside the heliosphere may be lower than the galactic intensity because of modulation in the local interstellar medium (5–7). Recent models indicate a reduction of ~25 to ~40% in the intensity of 100-MeV protons, with corresponding positive radial gradients of ~0.5 and ~0.9%/AU near the heliopause (7), although other models suggest that there should be no interstellar gradient (8). The time dependence of the observed intensities after the disappearance of heliospheric protons (DOY 270 to 366 in Fig. 1) corresponds to gradients of $-1.4 \pm 0.9\%/AU$ for 7- to 60-MeV protons and $-1.0 \pm 0.4\%/AU$ for >70-MeV GCR nuclei, mainly protons. The gradient of 6- to 100-MeV electrons is also small, only $-0.6 \pm 0.6\%/AU$ from DOY 239 to 366. Thus, there is no evidence for a positive radial gradient in the current region.

The GCR H and He spectra have the same shape from ~3 to 346 MeV per nucleon. The H/He ratio has been determined in three energy ranges corresponding to different telescope and/or operation modes of the instrument. In the lowest energy band, 3 to 7.8 MeV per nucleon, we find the H/He ratio to be 11.9 ± 0.4 . In the 7.8- to 57 MeV per nucleon band, the ratio is 12.9 ± 0.6 , and in the highest energy interval, 134 to 346 MeV per nucleon, the ratio is 12.6 ± 0.3 . These uncertainties are purely statistical, and there may be systematic uncertainties as well. However, the reasonably good agreement of the ratios across the range from 3 to 346 MeV per nucleon suggests that systematic uncertainties are likely small. The peak intensities of the GCR spectra of H and He are in the ~10- to 40-MeV per nucleon energy range.

The H/He ratio of 12.9 ± 0.6 in the energy region of the peak in the spectra is consistent with the recommended abundance in the solar photosphere, 12.6 (9). It differs from previous cosmic-ray observations of 4.7 ± 0.5 at 100 MeV per nucleon observed at 1 AU, where the spectra and abundance ratios are modified by the effects of solar modulation (10). It also differs from the ACR ratio of 4.1 (11), indicating that ACRs do not dominate GCRs outside the heliosphere as has been suggested (12).

The leaky-box model of Ip and Axford (13) addresses the low-energy portion of the GCR spectra (Fig. 3). Their model appears to have about the right peak intensity for H, but the energy of the peak is lower than observed. For He, both the peak intensity and the energy of the peak are somewhat displaced from the observations. At higher energies, >70 MeV per nucleon, the leaky-box model spectra from Webber and Higbie (14) are in good agreement with the observed H and He spectra.

Also shown in Fig. 3 are C and O spectra for 2012/261 to 366. This longer period improves the statistical significance of the observations and is justified by the intensity-versus-time profile of O with 5.4 to 13.9 MeV per nucleon for LETs A, B, and C shown in Fig. 2. In constructing the energy spectra for O in Fig. 3, the LET D telescope was not used, because it has a more persistent heliospheric contribution resulting from its bore sight looking nearly perpendicular to the magnetic field direction. Even ignoring LET D, there may be some residual heliospheric contribution present below ~10 MeV per nucleon. The C and O energy spectra above ~10 MeV per nucleon are very similar, with the peak C intensity occurring at ~70 MeV per nucleon. The C/O ratio for the energy range 21.6 to 106 MeV per nucleon is 0.95 ± 0.06 , consistent with GCR observations at higher energies at 1 AU (10, 15) and not with the ACR ratio of 0.005 (11). All previous measurements below ~100 MeV per nucleon represent particles decelerated from much higher energies by the solar modulation process.

The intensity of electrons with ~6 to 100 MeV had jumps in concert with the GCR nuclei (Fig. 1), indicating that V1 is observing GCR electrons in this energy range as opposed to electrons accelerated in the heliosphere. The ratio of intensities of the two periods shown in Fig. 4 is roughly a factor of two over the energy range shown, indicating an energy-independent diffusive mean free path for 6- to 60-MeV electrons.

The energy spectrum for the new region is proportional to $E^{-1.45 \pm 0.09}$ and has a spectral shape that is very similar to the theoretical estimates of the interstellar electron spectrum of Ip and Axford (13) and Langner *et al.* (16), but the overall intensity is about a factor of two below those estimates. The observed intensity exceeds an extrapolation of the spectrum from a diffusion model by Strong *et al.* (17) that assumes an electron source spectrum proportional to $E^{-1.6}$ below a few GeV, as implied by fits to radio synchrotron emission. A source spectrum of E^{-2} would better match the electron spectrum (Fig. 4) but would not be consistent with the radio observations without other adjustments to the model.

The presence of a region having a spiral magnetic field, but depleted of energetic heliospheric particles and accessible by low-energy GCR nuclei and electrons, is an important feature of the interaction between the heliosphere and the local interstellar medium. It has been suggested that

this could be a disconnection region where the spiral field has been convected far enough beyond the termination shock so that there is not an effective connection to the source of anomalous cosmic rays at the termination shock (18). Further development of this and other possible models will benefit our understanding of the region beyond 122 AU that Voyager 1 is now exploring.

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Acknowledgments: This work was supported by NASA (NNN12A012). This paper is dedicated to the memory of Frank McDonald, whose leadership in the cosmic-ray investigation on Voyager began in 1972. His contributions continued until the day of his passing, just after Voyager 1 durably entered the depletion region and fulfilled his vision of observing low-energy galactic cosmic rays from the local interstellar medium. This paper benefited substantially from discussions during meetings of the International Team on the Physics of the Heliopause at the International Space Science Institute in Bern, Switzerland.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1236408/DC1
Supplementary Text

Fig. S1

References

11 February 2013; accepted 24 May 2013
Published online 27 June 2013;
10.1126/science.1236408

One-Step Assembly of Coordination Complexes for Versatile Film and Particle Engineering

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The development of facile and versatile strategies for thin-film and particle engineering is of immense scientific interest. However, few methods can conformally coat substrates of different composition, size, shape, and structure. We report the one-step coating of various interfaces using coordination complexes of natural polyphenols and Fe(III) ions. Film formation is initiated by the adsorption of the polyphenol and directed by pH-dependent, multivalent coordination bonding. Aqueous deposition is performed on a range of planar as well as inorganic, organic, and biological particle templates, demonstrating an extremely rapid technique for producing structurally diverse, thin films and capsules that can disassemble. The ease, low cost, and scalability of the assembly process, combined with pH responsiveness and negligible cytotoxicity, makes these films potential candidates for biomedical and environmental applications.

Advances in materials design and application are highly dependent on the development of versatile thin-film and particle engineering strategies (1–4). Supramolecular metal-organic thin films have attracted widespread interest due to their diverse properties, which include (i) stimuli responsiveness imparted by the dynamic nature of supramolecular coordination bonds, (ii) hybrid physicochemical properties of both metals and organic materials, and (iii) controlled structure and functionality achieved by variation of the molecular building blocks (5, 6). Although metal-organic thin films show potential for sensing, separation processes, and catalysis (5, 6), such films are fabricated with multiple,

time-consuming steps (7–11). Moreover, biomedical applications of these films have thus far been limited because they can be toxic or unstable in water (12).

We report a simple, rapid, and robust conformal coating method using the one-step assembly of coordination complexes on a range of substrates to prepare various films and particles. The natural polyphenol tannic acid (TA) and Fe^{III} were chosen as the organic ligand and the inorganic cross-linker, respectively. Film deposition occurs upon mixing TA and Fe^{III} in water at ambient temperature. No special equipment is necessary for this method, and the material components are readily available and inexpensive. Moreover, they are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Although the chemical structure of TA is usually given as a decagalloyl glucose ($\text{C}_{76}\text{H}_{52}\text{O}_{46}$) (fig. S1) (13), it is actually a mixture of polygalloyl

glucose molecules with different degrees of esterification (14). Three galloyl groups from TA can react with each Fe^{III} ion to form a stable octahedral complex (15), allowing each TA molecule to react with several Fe^{III} centers to form a cross-linked film. This method is applicable to a wide variety of substrates because of the general surface binding affinity of TA. When these films are deposited on particles, subsequent dissolution of the templates results in the formation of three-dimensional free-standing films known as hollow capsules. Such systems have widespread use in drug and gene delivery, catalysis, and biosensing; they can also function as microreactors (16).

We first describe the deposition of Fe^{III} -TA films on planar (Fig. 1A) and particulate (Fig. 1, B to K) polystyrene (PS) templates. The color of the template suspension immediately turned blue upon addition of Fe^{III} and TA solutions (fig. S2). Stirring times (20 s and 1 hour) had no effect on the color or on the resulting film thickness under standard conditions (13), implying that the film formation process was completed instantaneously. Formation of the Fe^{III} -TA films on the PS particles shifted the surface zeta potential from -27 ± 3 mV to -64 ± 7 mV because of the acidic nature of the galloyl groups in TA. After removing the PS template, we obtained highly uniform microcapsules with a zeta potential of -65 ± 7 mV, which was within error the same value as before template removal.

Differential interference contrast (DIC) microscopy, atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) images of the Fe^{III} -TA capsules obtained from $D = 3.6$ μm templates are shown in Fig. 1, B to E. Monodisperse, spherical capsules were readily observed under DIC (Fig. 1B). The permeability of these capsules is molecular weight-dependent and they are essentially impermeable to 2000-kD dextran (fig. S3).

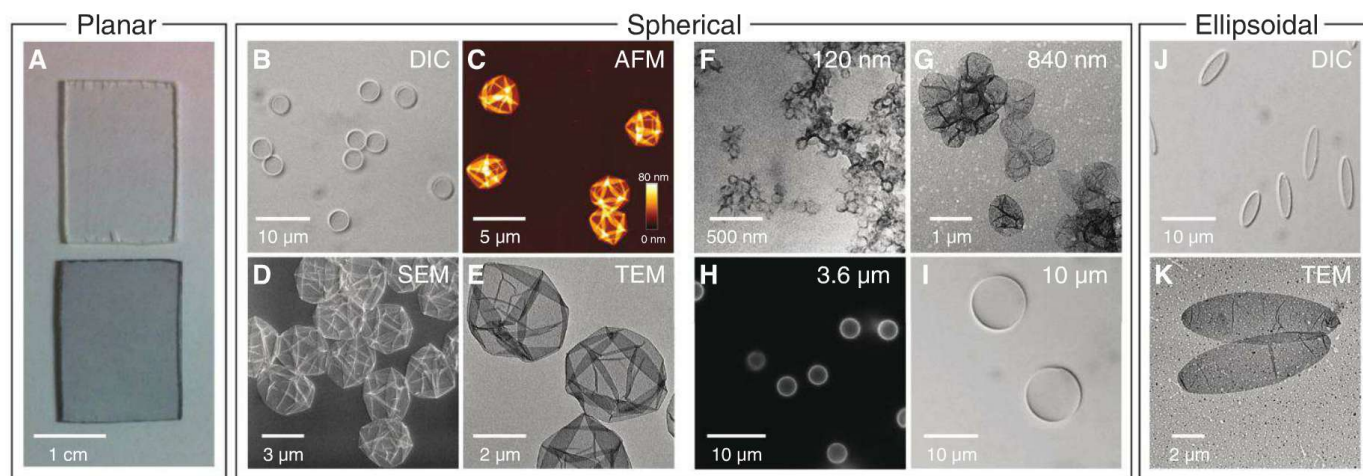


Fig. 1. Film formation. Fe^{III} -TA films prepared on PS substrates with various shapes (planar, spherical, and ellipsoidal) and sizes ($D = 120$ nm to 10 μm). (A) Photograph of PS slides before (top) and after (bottom) Fe^{III} -TA coating.

(B to K) Microscopy images of Fe^{III} -TA capsules: DIC images [(B), (I), and (J)], AFM images (C), TEM images [(E), (F), (G), and (K)], SEM image (D), and fluorescence microscopy image (H).

The presence of Fe in the films was confirmed by energy-dispersive x-ray spectroscopy (EDS) (fig. S4) and x-ray photoelectron spectroscopy (XPS) (fig. S5). The capsules observed by AFM, SEM, and TEM (Fig. 1, C to E) had folds and creases because these measurements were performed on dried samples. Different-sized templates ($D = 120$ nm, 840 nm, 3.6 μm , and 10 μm) can be exploited for capsule preparation (Fig. 1, F to I). Ellipsoidal PS templates, prepared by stretching spherical PS particles above their glass transition temperature, were also used to obtain ellipsoidal capsules (Fig. 1, J and K). From AFM height analysis, the single-wall thickness of the capsules was determined to be half the minimum height of the collapsed flat regions (10.4 ± 0.6 nm). The Young's modulus (E_Y) of the $D = 3.6$ μm PS-templated capsules was estimated to be 1.0 ± 0.2 GPa by AFM force measurements (fig. S6). This value of E_Y is at the high end of the range observed for layer-by-layer (LbL) polyelectrolyte capsules (10 to 1000 MPa) (17). Several groups have reported LbL capsules fabricated from TA and other polymers (18, 19). For example, LbL capsules of TA-poly(*N*-vinylpyrrolidone) (PVPON) exhibit a bilayer thickness of 1.0 to 2.2 nm, depending on the molecular weight of the PVPON (19). The Fe^{III} -TA film obtained through one-step assembly is thicker than four bilayers of TA-PVPON obtained through multistep LbL assembly, demonstrating the efficiency of the one-step process. Analogous to LbL assembly, the thickness of Fe^{III} -TA films can be further increased by simply repeating the rapid coating procedure (figs. S7 and S8).

The effect of TA and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ concentrations (hereafter denoted [TA] and $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$, respectively) on the resulting film thickness and

morphology were investigated by AFM using $D = 3.6$ μm PS-templated capsules. When [TA] was kept constant at 0.40 mg ml^{-1} (0.24 mM), capsules were obtained over a $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$ range of 0.06 to 0.20 mg ml^{-1} (0.22 to 0.74 mM), approximately corresponding to molar ratios of 1:1 to 3:1 between Fe^{III} and TA. The resulting stoichiometries in the capsule walls were determined by XPS (fig. S5) to be $\text{Fe}^{\text{III}}:\text{TA} \approx 1:4$, $1:3$, and $1:2$ for feed $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$ of 0.06 , 0.12 , and 0.20 mg ml^{-1} , respectively. This demonstrates that the feed concentrations influence the capsule stoichiometries. Above and below $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}] = 0.06$ to 0.20 mg ml^{-1} , aggregated capsules and few capsules were formed, respectively. In the absence of Fe^{III} , no capsules were formed. As $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$ was increased in this concentration range, the thickness of the Fe^{III} -TA film increased from 7.7 ± 0.4 nm to 11.9 ± 1.2 nm and exhibited increased roughness (fig. S9). Increasing the amount of Fe^{III} to three molar equivalents of TA resulted in capsules that had a grainy surface because of the excess Fe^{III} (fig. S9A). Values of root-mean-square roughness (300 nm $\times$ 300 nm, fold-free flat region) were 1.3 ± 0.1 nm, 1.6 ± 0.1 nm, and 7.7 ± 0.4 nm for $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$ of 0.06 , 0.10 , and 0.20 mg ml^{-1} , respectively. In contrast to these observations based on Fe^{III} , [TA] had minimal impact upon film assembly. Capsules with constant film thickness and roughness were obtained throughout a concentration range of [TA] = 0.10 to 1.80 mg ml^{-1} (0.06 to 1.06 mM) when $[\text{FeCl}_3 \cdot 6\text{H}_2\text{O}]$ was fixed at 0.10 mg ml^{-1} (0.37 mM) (fig. S10). These results suggest that TA was relatively in higher excess than Fe^{III} under these conditions, and that Fe^{III} , not TA, influenced the film thickness.

To further investigate the mechanism of the Fe^{III} -TA film formation, we used polyethyleneimine

(PEI)-coated PS templates for capsule preparation. The PEI coating changed the zeta potential of the templates from negative (-27 ± 3 mV) to positive (37 ± 6 mV). Capsules were still formed with these positively charged templates, indicating that the surface charge is not an important factor for film deposition. We also determined the zeta potential after incubating the bare PS particles with either TA or FeCl_3 . The adsorption of TA reduced the zeta potential to -39 ± 4 mV, whereas the value was slightly changed after incubation with FeCl_3 (-33 ± 5 mV). Rapid surface adherence and formation of a TA layer on the template particle surface were confirmed by adsorption experiments (fig. S11). Catechol-functionalized molecules and their derivatives have a high affinity for a wide variety of substrates with different surface charges (4, 20). Thus, it is most likely that the free TA or small Fe^{III} -TA complexes initially adsorb onto the template surface and are subsequently cross-linked by further Fe^{III} complexation. The increase in the cross-linker concentration causes the attraction of more TA to the initially formed films, making them thicker. Film growth is completed when free Fe^{III} in the bulk solution is consumed. Excess Fe^{III} induces aggregation of Fe^{III} -TA complexes in the bulk solution. These small aggregates subsequently bind to the surface, leading to an increased roughness of the capsule films (fig. S12). Unlike thin-film formation using dopamine self-polymerization (4), this study relies on the complexation of TA with Fe^{III} through coordination bonds, which allows the films to form rapidly and disassemble in response to pH (see below).

To show the versatility of this method, we coated various planar and particulate substrates exhibiting different surface properties (anionic,

Fig. 2. Fe^{III} -TA coating on various substrates.

(A to C) Photograph of planar substrates before (upper) and after (lower) Fe^{III} -TA coating: glass (A), Au (B), and PDMS (C). (D) Zeta potential values of particulate substrates in water before and after Fe^{III} -TA coating. Data are means $\pm$ SD. (E) Confocal laser scanning microscopy image of protein-loaded CaCO_3 particles (red) coated with Fe^{III} -TA films (green). (F and G) TEM images of Au NPs noncoated (F) and coated (G) with a Fe^{III} -TA film. (H and I) Photographs of Fe^{III} -TA capsules loaded with Fe_3O_4 nanoparticles dispersed in water before (H) and after (I) applying a magnet.

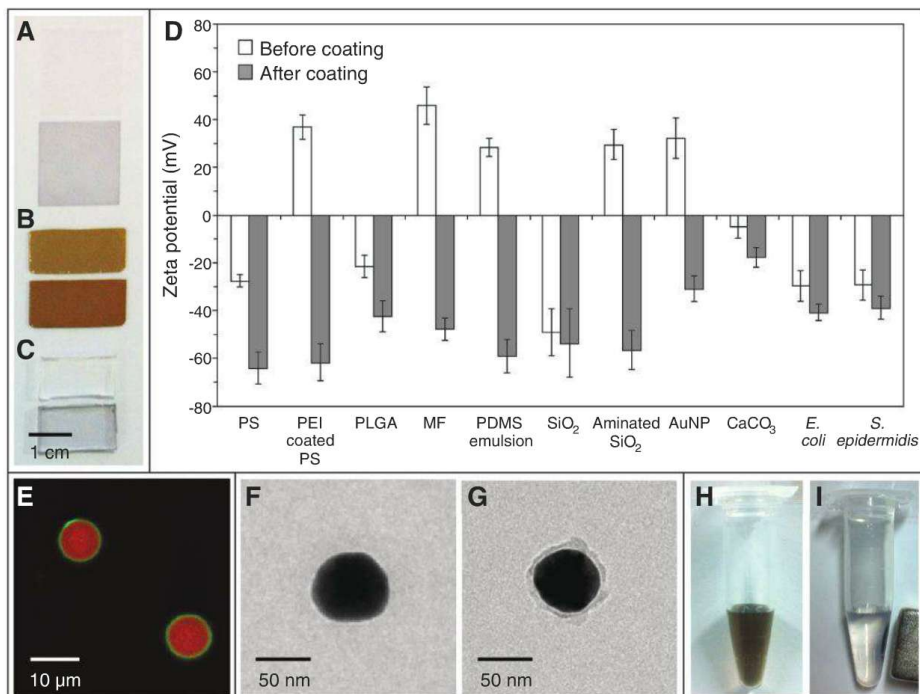
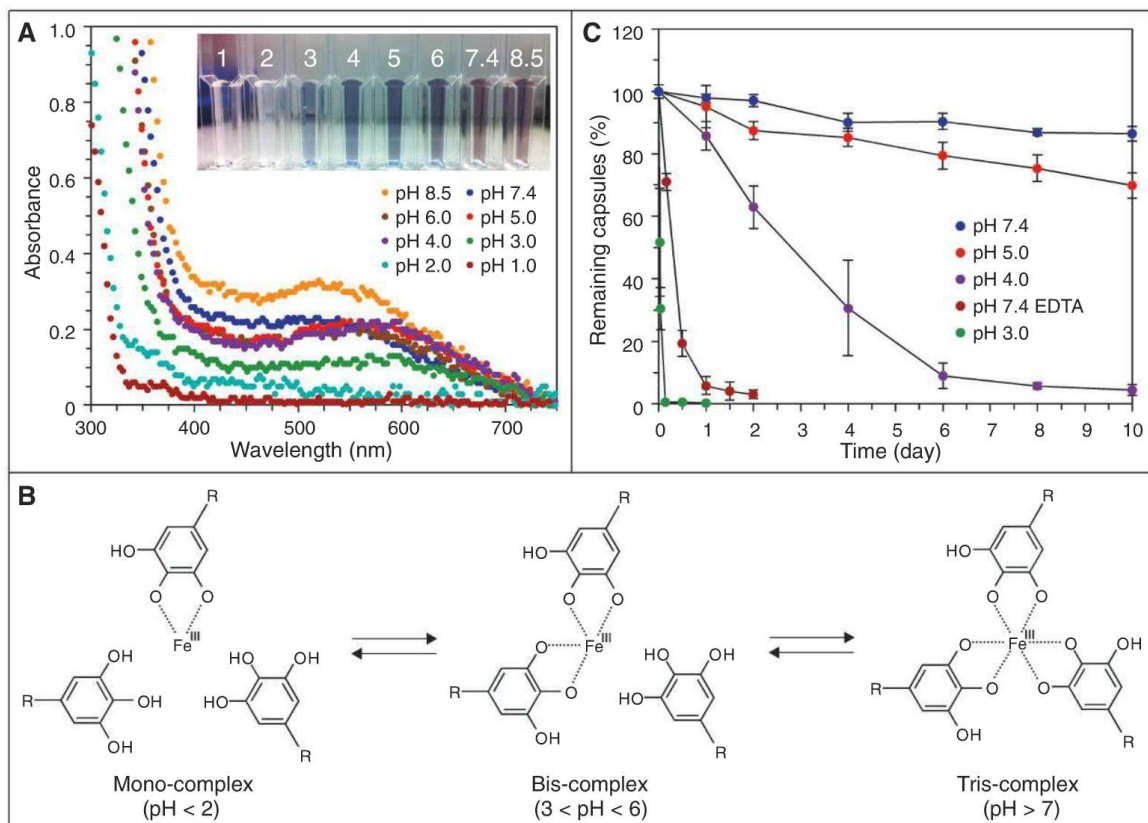


Fig. 3. pH-responsive disassembly of Fe^{III}-TA capsules. (A) Ultraviolet to visible absorption spectra of spherical Fe^{III}-TA capsule dispersions ($D = 3.6 \mu\text{m}$, 4.0×10^7 capsules mL^{-1}) at various pH values. Inset is a photograph of the capsule dispersions at the indicated pH values. (B) pH-dependent transition of dominant Fe^{III}-TA complexation state. R represents the remainder of the TA molecule. (C) Plot of remaining capsule population versus time. Data are means $\pm$ SD from three independent measurements.



neutral, and cationic)—including glass, gold (Au), polydimethylsiloxane (PDMS), poly(lactic-co-glycolic acid) (PLGA), melamine-formaldehyde resin, low-molecular weight PDMS emulsion, silica (SiO₂), aminated SiO₂, cetyltrimethylammonium bromide-capped Au nanoparticles (Au NPs), calcium carbonate (CaCO₃), *Escherichia coli*, and *Staphylococcus epidermidis*—with the Fe^{III}-TA films (Fig. 2). The color and zeta potential values (Fig. 2, A to D) changed after the coating in all cases, demonstrating that Fe^{III}-TA films can be formed on a wide variety of substrates. Figure 2E and fig. S13A show protein- and rhodamine B-loaded CaCO₃ coated with Fe^{III}-TA films, respectively. Replica particles were obtained by filling mesoporous CaCO₃ particles with Fe^{III}-TA complexes and dissolving the CaCO₃ cores (fig. S13, B to E). After the coating, a Fe^{III}-TA shell layer was visible around the Au NP core (Fig. 2, F and G, and fig. S13F). Magnetic Fe₃O₄ nanoparticles were encapsulated by coating low-molecular weight PDMS emulsion templates loaded with Fe₃O₄ nanoparticles (fig. S13G). Subsequent removal of the emulsion by ethanol produced magnetically active Fe^{III}-TA capsules (Fig. 2, H and I).

The coordination between Fe^{III} and TA is pH-dependent, and the obtained capsules exhibit pH-dependent disassembly. The color of the capsule suspension was also pH-dependent (Fig. 3A). The suspension was colorless at pH < 2, blue at 3 < pH < 6, and red at pH > 7. This color change is consistent with observations for analogous Fe^{III}-

catechol complexes (21, 22), which can be attributed to transitions between mono-, bis-, and tris-complex states (Fig. 3B). At pH 2.0, the Fe^{III}-TA capsules shrank immediately (fig. S14) and disassembled. At low pH, most of the hydroxyl groups were protonated, which led to rapid destabilization of cross-links and disassembly of the films. Even above pH 3.0, Fe^{III}-TA capsules disassembled. Figure 3C shows the disassembly kinetics of the Fe^{III}-TA capsules. At pH 3.0, all of the capsules had disassembled in 4 hours, whereas at pH 4.0, 6 days of incubation were required to disassemble the majority of the capsules. In contrast, ~70 and 90% of the capsules still remained intact after 10 days of incubation at pH 5.0 and pH 7.4, respectively. The stability constants of Fe^{III}-TA are 1.5×10^5 , 3.4×10^9 , and 2.8×10^{17} at pH 2, 5, and 8, respectively (23). Additionally, we carried out AFM experiments after incubation at pH 5.0 (fig. S15). The films became thinner (6.0 ± 1.4 nm) and rougher, confirming the gradual disassembly of the Fe^{III}-TA films. Ethylenediaminetetraacetic acid (EDTA) accelerated the disassembly because of its strong affinity for Fe^{III} (Fig. 3C).

The cytotoxicity of Fe^{III}-TA capsules was found to be negligible (fig. S16). Coupled with their pH-sensitive disassembly profile, Fe^{III}-TA capsules have potential biomedical application because of the varying pH in different parts of the body [e.g., blood (pH 7.4), stomach (pH 1.0 to 3.0), duodenum (pH 4.8 to 8.2), etc.] (24). Iron tannate has been historically recognized as an ink

(25) and a corrosion-resistant layer for steel (15). It had also been used for dyeing teeth black, and consequently preventing cavities, in the old Japanese custom called *ohaguro* (26). The general applicability of this technique was further demonstrated by using different metals and another polyphenol (figs. S17 and S18). The simple preparation and biologically tunable physicochemical properties of the metal-polyphenol films provide a platform for the engineering and assembly of advanced materials for potential use in a range of applications.

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Acknowledgments: Supported by the Australian Research Council under Federation Fellowship FF0776078 (F.C.), Australian Laureate Fellowship FL120100030 (F.C.), Discovery Project DP0877360 (F.C.), Future Fellowship FT120100564 (G.K.S.), and Super Science Fellowship FS110200025 (J.C. and F.C.) schemes. H.E. thanks the Japan Society for the

Promotion of Science (JSPS) for a postdoctoral fellowship for research abroad. The data presented in this paper are given in the main text and in the supplementary materials. We thank X. Duan (Surface and Chemical Analysis Network, the University of Melbourne) for assistance with XPS analysis. The University of Melbourne has filed a provisional patent on the assembly process.

Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6142/154/DC1

Materials and Methods

Figs. S1 to S18

References (27–30)

1 March 2013; accepted 5 June 2013

10.1126/science.1237265

Nanoscale Atoms in Solid-State Chemistry

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We describe a solid-state material formed from binary assembly of atomically precise molecular clusters. $[\text{Co}_6\text{Se}_8(\text{PET}_3)_6][\text{C}_{60}]_2$ and $[\text{Cr}_6\text{Te}_8(\text{PET}_3)_6][\text{C}_{60}]_2$ assembled into a superatomic relative of the cadmium iodide (CdI_2) structure type. These solid-state materials showed activated electronic transport with activation energies of 100 to 150 millielectron volts. The more reducing cluster $\text{Ni}_9\text{Te}_6(\text{PET}_3)_8$ transferred more charge to the fullerene and formed a rock-salt–related structure. In this material, the constituent clusters are able to interact electronically to produce a magnetically ordered phase at low temperature, akin to atoms in a solid-state compound.

Conventional binary solid-state compounds, A_xB_y , are infinite, crystalline arrays of atoms A and B. Here, we describe analogous binary solids in which the “atomic” building blocks are pseudo-spherical molecular clusters rather than simply atoms [for reviews on molecular clusters, see (1–3)]. We prepare these new solids by combining independently synthesized molecular clusters (4–6). The internal structures of the constituent clusters remain unchanged, but charge is transferred between them, forming ionic solids analogous to NaCl . We report three new solids: $[\text{Co}_6\text{Se}_8(\text{PET}_3)_6][\text{C}_{60}]_2$, $[\text{Cr}_6\text{Te}_8(\text{PET}_3)_6][\text{C}_{60}]_2$,

and $[\text{Ni}_9\text{Te}_6(\text{PET}_3)_8][\text{C}_{60}]$. The former two assemble into a superatomic relative of the CdI_2 structure type, and the latter forms a simple rock-salt crystal.

Despite their ready availability, molecular clusters have been used infrequently as electronic materials. Noteworthy examples of success in this area are the organic-inorganic hybrid materials reported by Batail and Mitzi (7–11). Nanocrystals have been assembled into striking superlattices (12–14), but they do not have discrete structural, electronic, and magnetic properties and cannot be regarded as genuine artificial atoms. Here,

we combine independently prepared electronically and structurally complementary molecular cluster building blocks to form atomically precise binary solid-state compounds. When the building blocks are atoms (ions), binary solids assemble into simple crystalline arrays such as the rock-salt and CdI_2 lattices [for an authoritative text on solid-state inorganic chemistry, see (15)]. We show that when similarly sized clusters combine, the same lattice results, albeit at the dramatically increased length scale of nanometers rather than Ångströms. The constituent clusters interact to produce collective properties such as electrically conducting networks and magnetic ordering.

Our strategy was to use constituent molecular clusters that have the same, roughly spherical, shape but very different electronic properties to encourage reaction and subsequent structural

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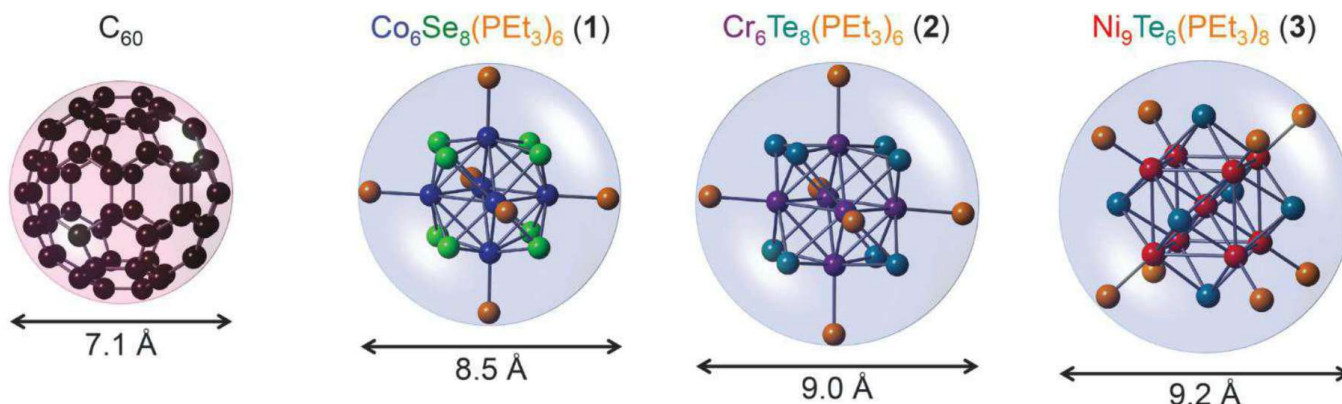


Fig. 1. Structures of the nanoscale atoms as measured by SCXRD. In the figure, the clusters are depicted on the same size scale. The diameter of the cluster is determined as the long diagonal P–P distance. The ethyl groups on the phosphines of **1**, **2**, and **3** were removed to clarify the view.

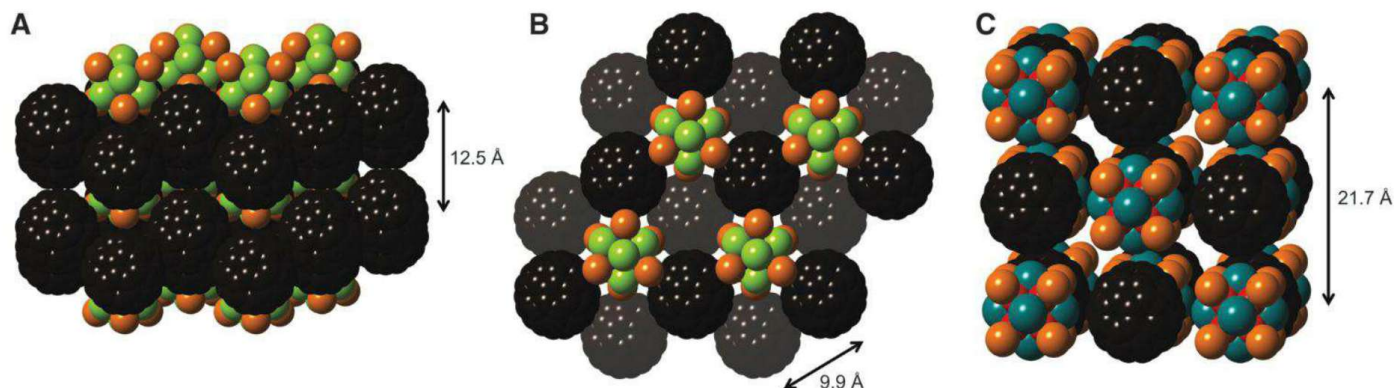


Fig. 2. Structures of solid-state compounds assembled from nanoscale atoms. Space-filling structure of $1 \cdot 2C_{60}$ showing the crystal packing looking along (A) the ab plane and (B) down the c axis. (C) Space-filling struc-

ture of $3 \cdot C_{60}$. Carbon, black; nickel, red; cobalt, blue; phosphorus, orange; tellurium, teal; selenium, green. The ethyl groups on the phosphines were removed to clarify the view.

association. By analogy to “atomic” solid-state chemistry, we reasoned that the in situ transfer of charge would produce ions (or the equivalent) that could then form an ordered solid. Thus, we sought cluster pairs in which one cluster is relatively electron-poor and the other is relatively electron-rich. C_{60} carbon clusters are good electron acceptors (16). The electrically neutral metal chalcogenide clusters $Co_6Se_8(PEt_3)_6$ (**1**), $Cr_6Te_8(PEt_3)_6$ (**2**), and $Ni_9Te_6(PEt_3)_8$ (**3**) are all electron-rich. Importantly, these clusters (Fig. 1) are similar in size and shape to the fullerene.

We combined **1** and two equivalents of C_{60} in toluene and obtained black crystals after ~12 hours. Single-crystal x-ray diffraction (SCXRD) revealed that this solid is a 1:2 stoichiometric combination of **1** and C_{60} ($1 \cdot 2C_{60}$) (Fig. 2, A and B) composed of hexagonal arrays of C_{60} s in a chairlike arrangement separated by layers of the clusters. The C_{60} layers are 12.5 Å apart. The centroid-to-centroid distance and the shortest nonbonded C-C spacing between two adjacent C_{60} s are 9.9 Å and 3.4 Å, respectively. These distances are comparable to crystalline C_{60} (17). We obtain the exact same structure when we combine the $Cr_6Te_8(PEt_3)_6$ cluster **2** and two equivalents of C_{60} in toluene (figs. S2 and S3) (18).

We measured how much charge was transferred between the components in the solid-state material using Raman spectroscopy. The pentagonal pinch mode of C_{60} (A_{2g}) (1468 cm^{-1} for pristine C_{60}) shifts to lower energy by 6 cm^{-1} per electron transferred to C_{60} , independent of the dopant or the crystal structure [see, for example, (19); for a review on discrete fulleride anions, see (20)]. The solid-state Raman spectra of $1 \cdot 2C_{60}$ and $2 \cdot 2C_{60}$ (fig. S4) (18) were taken using a 514.5-nm excitation laser at 4.6 to 7.8 kW/cm^2 power densities. The A_{2g} modes of C_{60} were centered at 1463 cm^{-1} and 1462 cm^{-1} in $1 \cdot 2C_{60}$ and $2 \cdot 2C_{60}$, respectively. The difference between the A_{2g} peak position of $1 \cdot 2C_{60}$ and $2 \cdot 2C_{60}$ is small and within experimental error. We estimate that clusters **1** and **2** transfer two electrons, and each C_{60} receives one electron.

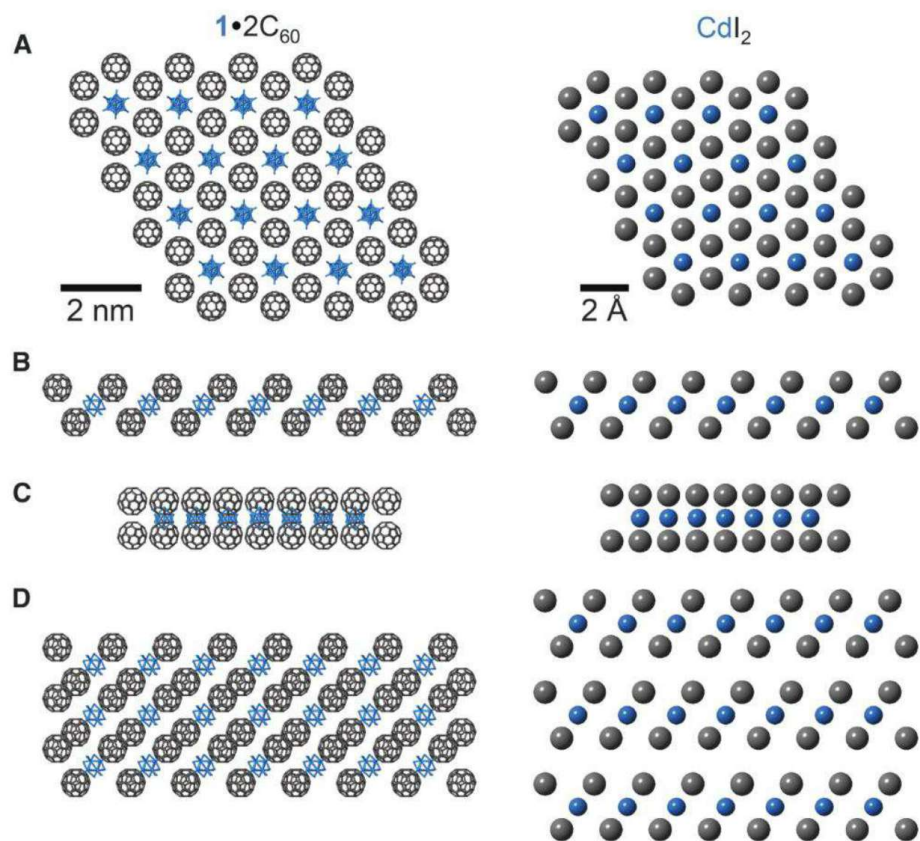


Fig. 3. Comparison of the packing structures of $1 \cdot 2C_{60}$ and CdI_2 . (A) View of a single hexagonal layer looking down the c axis. (B) and (C) Edge-on views of the same layer looking along the ab plane. (D) Stacking of the ab hexagonal layers along the c -axis direction. Cluster **1** and Cd are shown in blue; C_{60} and I are shown in gray. The ethyl groups on the phosphines were removed to clarify the view.

The solid-state electronic absorption spectra of $1 \cdot 2C_{60}$ and $2 \cdot 2C_{60}$ provide additional confirmation for the formation of charge-transfer complexes in the materials. The electronic spectrum of each material dispersed in a KBr pellet shows a series of transitions between 900 and 1150 nm with the strongest band centered at 1100 nm (figs. S6 and S7) (18). These features are transitions for the radical anion of fullerene, $C_{60}^{\cdot -}$ (20).

Cluster **1** has four weak transitions between 350 and 700 nm that were observed in $1 \cdot 2C_{60}$ but not in $2 \cdot 2C_{60}$.

We can compare these solids to traditional simple $M^{2+}X^{1-}_2$ solids. The CdI_2 structure type (21) is formed by a hexagonally close-packed array of monoanions with half of the octahedral interstitial sites occupied by dications. The cations are ordered such that along the crystallographic

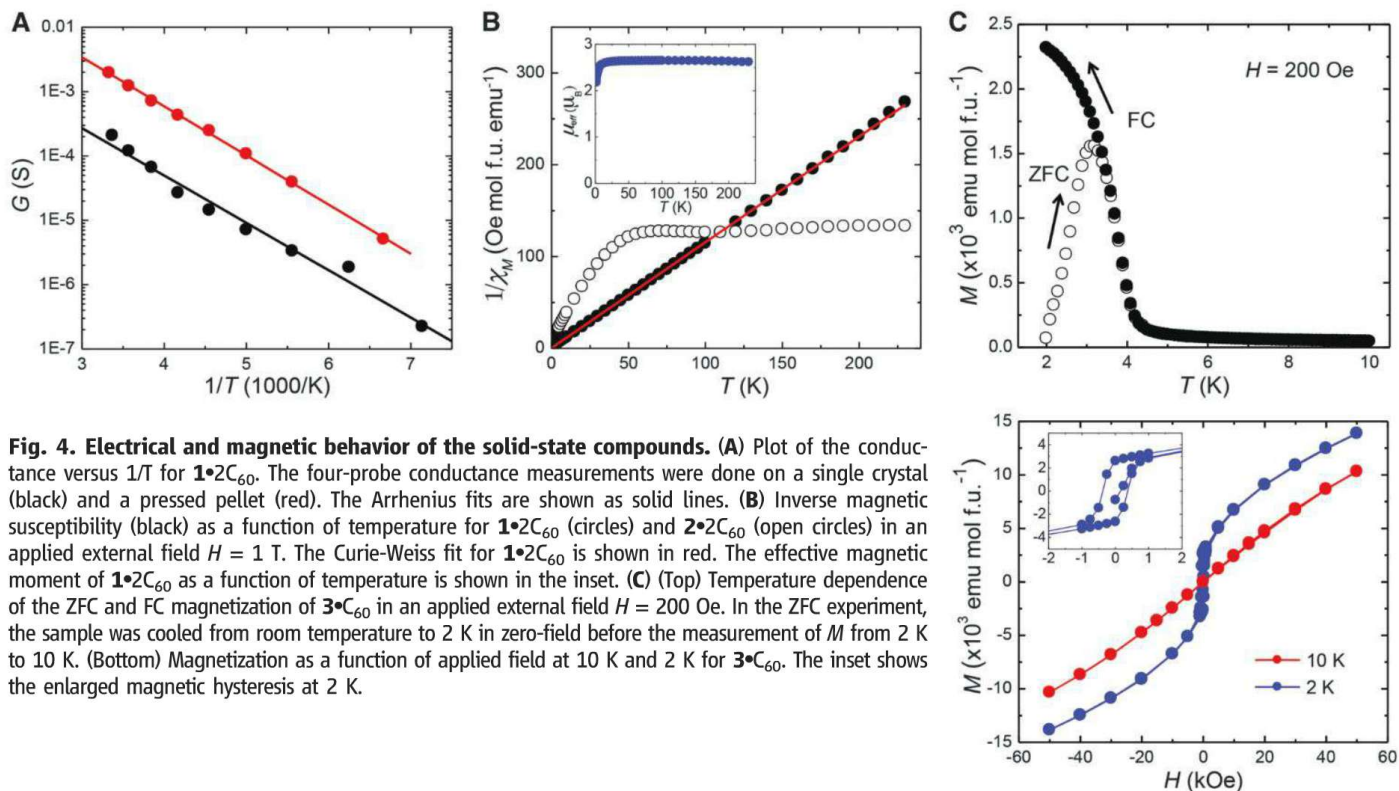


Fig. 4. Electrical and magnetic behavior of the solid-state compounds. (A) Plot of the conductance versus $1/T$ for $1\bullet 2C_{60}$. The four-probe conductance measurements were done on a single crystal (black) and a pressed pellet (red). The Arrhenius fits are shown as solid lines. (B) Inverse magnetic susceptibility (black) as a function of temperature for $1\bullet 2C_{60}$ (circles) and $2\bullet 2C_{60}$ (open circles) in an applied external field $H = 1$ T. The Curie-Weiss fit for $1\bullet 2C_{60}$ is shown in red. The effective magnetic moment of $1\bullet 2C_{60}$ as a function of temperature is shown in the inset. (C) (Top) Temperature dependence of the ZFC and FC magnetization of $3\bullet C_{60}$ in an applied external field $H = 200$ Oe. In the ZFC experiment, the sample was cooled from room temperature to 2 K in zero-field before the measurement of M from 2 K to 10 K. (Bottom) Magnetization as a function of applied field at 10 K and 2 K for $3\bullet C_{60}$. The inset shows the enlarged magnetic hysteresis at 2 K.

c direction, the cation layers are alternatively empty and fully occupied, and the layers are held together by van der Waals bonding between anions of neighboring layers. The structures of compounds $1\bullet 2C_{60}$ and $2\bullet 2C_{60}$ can be appreciated in these same terms. Wireframe representation of $1\bullet 2C_{60}$ are shown in Fig. 3; in Fig. 3A, we compare one C_{60} -cluster- C_{60} -layer to the corresponding layer in CdI_2 . In Fig. 3, B to D, we show edge-on and packing views of these same layers; the similarity between our cluster-solid and the “atomic” solid is evident. Although atomic solid CdI_2 appears in many different polytypes, which are related by different patterns of stacking of ab planes, we have observed only one stacking polytype in our cluster solids.

The cluster $Ni_9Te_6(PEt_3)_8$ (**3**) is rich in metal, and we expect this compound to have a greater reducing power than **1** or **2**. Interdiffusion of cluster **3** and C_{60} solutions for 2 weeks at -30°C gives a black solid that is composed of micron-sized cubic crystals. Rietveld refinement of the synchrotron powder x-ray diffraction data (fig. S1) (18) reveals a 1:1 combination of **3** and C_{60} ($3\bullet C_{60}$) (Fig. 2C) in a face-centered cubic structure analogous to rock salt with a lattice parameter of 21.7 Å. We observed a broad peak centered at 1454 cm^{-1} in the solid-state Raman spectrum of $3\bullet C_{60}$ (fig. S5) (18). We assign this peak to the A_{2g} mode of C_{60} , and these data strongly suggest that the fullerene in $3\bullet C_{60}$ is more reduced than in $1\bullet 2C_{60}$ or $2\bullet 2C_{60}$. These results show that we can prepare binary cluster materials with diverse structural and ionic proper-

ties by changing the composition of the molecular cluster building block.

These materials behave less like molecular co-crystals and more like three-dimensional solid-state compounds. For example, $1\bullet 2C_{60}$ and $2\bullet 2C_{60}$ exhibit activated electronic transport. Figure 4A displays the electrical transport properties of the co-crystals $1\bullet 2C_{60}$ (fig. S9 shows the transport properties of $2\bullet 2C_{60}$) (18). We performed two- and four-probe electrical resistivity measurements on single crystals and pressed pellets of $1\bullet 2C_{60}$ and two-probe measurements on pressed pellets of $2\bullet 2C_{60}$. Both compounds are good electrical conductors with resistivities on the order of 10 ohm-cm at room temperature. We observe an exponential decrease of the conductance (G) with decreasing temperature. This thermally activated semiconducting behavior displayed Arrhenius behavior with activation energies E_a of ~ 150 meV and ~ 100 meV for $1\bullet 2C_{60}$ and $2\bullet 2C_{60}$, respectively, and indicates that $1\bullet 2C_{60}$ and $2\bullet 2C_{60}$ are both gapped semiconductors.

An additional feature of these superatom-assembled solids is that the magnetic properties vary as the inorganic cores are changed because of the vastly different spin states accessible with the molecular clusters. Figure 4B shows the temperature dependence of the inverse molar magnetic susceptibility ($1/\chi_M$) and the effective magnetic moment (m_{eff}) of $1\bullet 2C_{60}$ from superconducting quantum interference device (SQUID) magnetometry. We corrected the data for diamagnetic and temperature-independent contributions and modeled the results using a modified Curie-Weiss law

$c_M(T) = \frac{C}{T-\theta} + c_D + c_{TIP}$, where C is the Curie constant, θ is the Weiss constant, and c_D and c_{TIP} are the diamagnetic and temperature-independent paramagnetic contributions, respectively. A good fit is obtained with $C = 0.9$ emu (electromagnetic unit) K Oe $^{-1}$ mol f.u. $^{-1}$ (formula unit), $\theta = -0.3$ K, and $c_{TIP} = 0.001$ emu Oe $^{-1}$ mol f.u. $^{-1}$. The small negative Weiss constant indicates weak antiferromagnetic interactions. Above 10 K, $1\bullet 2C_{60}$ showed a temperature-independent effective magnetic moment, $m_{eff} = 2.7\text{ }\mu_B$ per f.u. This result agrees well with the spin-only value of $2.8\text{ }\mu_B$ for two noninteracting unpaired electrons and is consistent with the Raman spectroscopy data that show one electron in each of the two C_{60} s per formula unit, with the cobalt ions in the cluster not contributing to the overall moment.

Figure 4B also displays the temperature dependence of the inverse molar magnetic susceptibility of $2\bullet 2C_{60}$. The important result is that $2\bullet 2C_{60}$ exhibits a more complex magnetic behavior, with a change in the slope of this plot around 60 K. We presume the difference in the materials is caused by the large magnetic difference between compound (**1**) $^{2+}$, which contains six Co III , and compound (**2**) $^{2+}$, which is composed of six Cr III .

The magnetism of the rock-salt $3\bullet C_{60}$ material is remarkably different from that of $1\bullet 2C_{60}$ and $2\bullet 2C_{60}$, both in magnitude and as a function of temperature. Figure 4C shows the temperature dependence of the magnetization (M) of $3\bullet C_{60}$. When we applied a field of 200 Oe and cooled the sample to 2 K, we measured no appreciable

magnetic response until the temperature reached about 4 K, at which point we observed a sudden transition to a magnetically ordered phase, with M reaching 2300 emu mol f.u.⁻¹ at 2 K. The difference between the zero-field cooled (ZFC) and field-cooled (FC) magnetizations indicates some irreversibility in the magnetically ordered phase. The magnetic response of $3\bullet\text{C}_{60}$ to an external field differs dramatically when the sample is examined at temperatures above the critical temperature (T_c) and below T_c (Fig. 4C). At $T = 10$ K, M scales linearly with the strength of the external applied magnetic field (H). The sigmoidal magnetization curve measured at 2 K is characteristic of ferromagnetism. Compound $3\bullet\text{C}_{60}$ exhibits a small hysteresis with a coercivity $H_c \sim 400$ Oe. This indicates that the spins freeze in a ferromagnetic state for $T < T_c$. This result demonstrates that the constituent clusters are able to communicate magnetically at low temperature in the same way that atoms are able to in solid-state compounds.

By using clusters that are similar in size and shape to each other, we have created binary assemblies whose infinite crystalline structures are determined not only by the shapes of the clusters but also by the degree of charge transfer between the constituents. The intercluster charge transfer, along with the intermolecular van der Waals interactions that are typical in conventional molecular solids, hold these solid-state compounds together in much the same way that interatomic charge transfer from Cd to I holds CdI_2 together and from Na to Cl holds rock salt together. These results chart a clear path to creating whole fam-

ilies of multifunctional solid-state materials whose electronic and magnetic properties can be tuned by varying the constitution of the superatom building blocks.

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Acknowledgments: This work was supported primarily through the Center for Re-Defining Photovoltaic Efficiency Through Molecular-Scale Control, an Energy Frontier Research Center (EFRC) funded by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under award DE-SC0001085. X.R. thanks the Natural Sciences and Engineering Research Council of Canada for a postdoctoral fellowship. C.-H.L. is partially supported by the Basic Science Research Program through the National Research Foundation of Korea (357-2011-1-C00035). C.L.S. is supported by the National Science Foundation Graduate Research Fellowship under award DGE-1144155. We thank Y. Uemura for the SQUID magnetometer. NHMFL was supported in part by the National Science Foundation Cooperative Agreement DMR-1157490, the State of Florida, the U.S. Department of Energy, and Florida State University. Use of the National Synchrotron Light Source, Brookhaven National Laboratory, was supported by the U.S. Department of Energy, Office of Basic Energy Sciences under contract DE-AC02-98CH10886. Structural analysis work done by T.B. and T.S. is supported by the U.S. Department of Energy, Office of Basic Sciences under contract DE-SC0008832, the State of Florida, and Florida State University. The crystallographic data presented in this work are available through the Cambridge Crystallographic Data Center referencing deposition nos. 940469 to 940472. We gratefully acknowledge R. J. Cava for very helpful conversations.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1236259/DC1
Materials and Methods
Figs. S1 to S9
Table S1
References (22–25)

7 February 2013; accepted 21 May 2013
Published online 6 June 2013;
10.1126/science.1236259

Fossil Musculature of the Most Primitive Jawed Vertebrates

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The transition from jawless to jawed vertebrates (gnathostomes) resulted in the reconfiguration of the muscles and skeleton of the head, including the creation of a separate shoulder girdle with distinct neck muscles. We describe here the only known examples of preserved musculature from placoderms (extinct armored fishes), the phylogenetically most basal jawed vertebrates. Placoderms possess a regionalized muscular anatomy that differs radically from the musculature of extant sharks, which is often viewed as primitive for gnathostomes. The placoderm data suggest that neck musculature evolved together with a dermal joint between skull and shoulder girdle, not as part of a broadly flexible neck as in sharks, and that transverse abdominal muscles are an innovation of gnathostomes rather than of tetrapods.

The most fundamental anatomical divide among living vertebrates is that between the jawless cyclostomes (lampreys and hagfishes) and the jawed gnathostomes (all other vertebrates). Although early development in the two groups is similar, differences in migration and proliferation patterns of cell populations produce profoundly different adult architectures in the head region, encompassing not only the skeleton

but also the musculature (*1*). In conjunction with the presence or absence of jaws, a major difference between the groups is the absence of a shoulder girdle, paired appendages, and a differentiated neck region in cyclostomes.

The gnathostome stem group contains jawless as well as jawed clades, suggesting that the jawless condition is primitive for vertebrates (*2*). Some jawless stem gnathostomes, such as osteo-

stracans, have pectoral fins and “shoulder girdles,” but the latter are immovably attached to the skull (*2*). Head-shoulder separation and the origin of distinctive neck muscles such as the cucullaris seem to have occurred at approximately the same time as the origin of jaws. The phylogenetically deepest and morphologically most primitive versions of jawed vertebrate anatomy are found in the placoderms, a paraphyletic array of Silurian-Devonian armored fishes that form the upper part of the gnathostome stem group (*3, 4*) (Fig. 1A).

Data on placoderm muscular anatomy would add considerably to the knowledge of gnatho-

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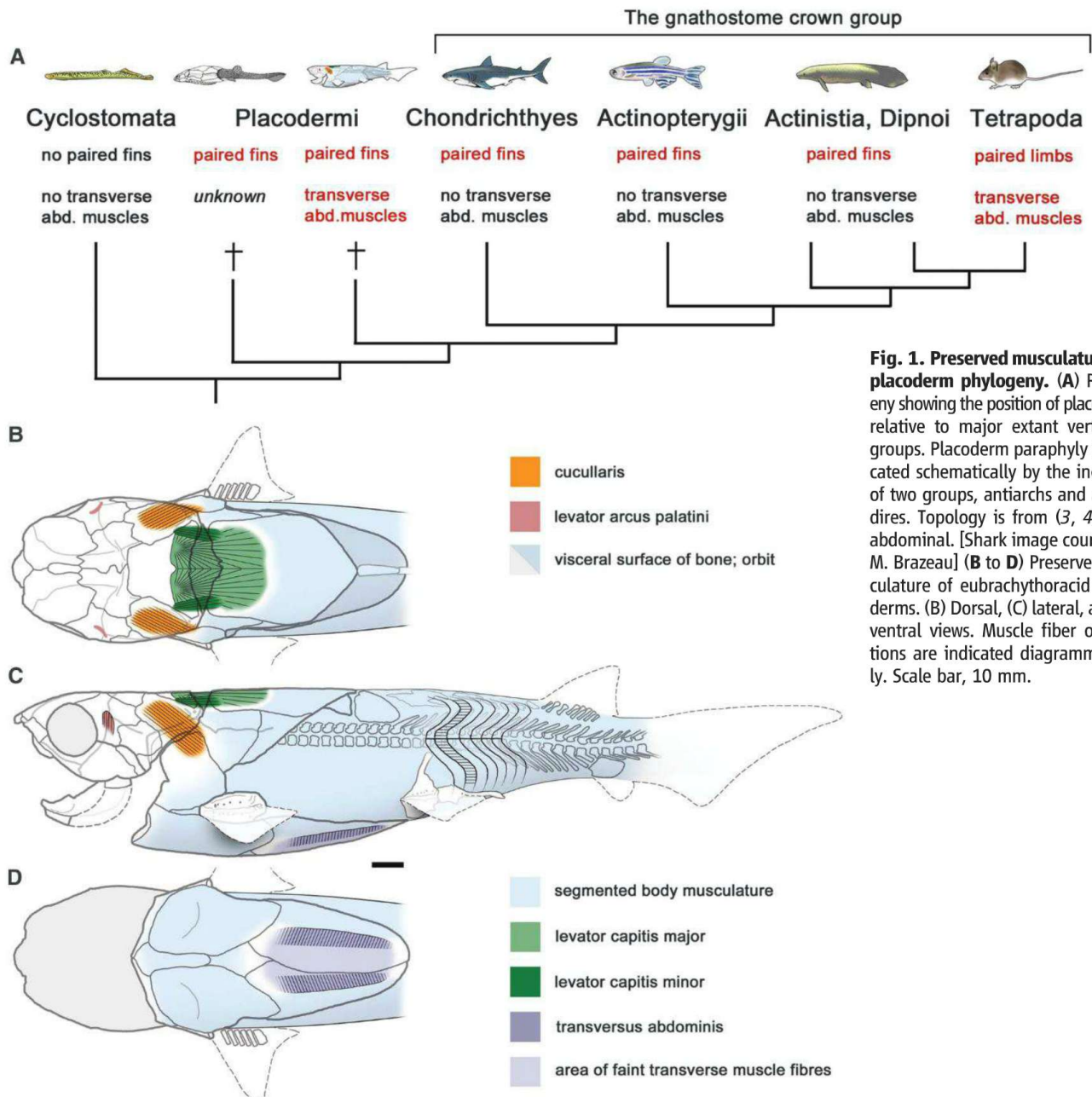
stome origins, given the functional and anatomical importance of the musculature in the transition from jawless to jawed vertebrates, as well as its importance as a probable indicator of cell population identity (5). Until now, such data have not been available, and conclusions about placoderm musculature have been based entirely on inferences from skeletal anatomy and functional morphology. Studies of muscular evolution in gnathostomes have often proceeded from the assumption that extant sharks are adequate models for the primitive gnathostome condition (6–8).

We present here placoderm fossils with preserved three-dimensional musculature, from the Upper Devonian Gogo Formation of Western Australia. The presence of neck and trunk muscles in

the eubranchyothoracid arthrodiroids *Eastmanosteus*, *Compagopiscis*, and *Incisoscutum* allows detailed reconstruction of this musculature (Figs. 1, B to D, 2, and 3 and figs. S1 and S2). Additional information about tail muscles comes from the ptyctodont *Austroptyctodus* (fig. S2, H and I). These specimens present an anatomy that differs radically from the shark model (6).

The presence of a dermal neck joint (Fig. 2A) between the skull and trunk armor of placoderms implies vertical pivoting movement of the head, requiring elevator and depressor muscles (9, 10). Such muscles are confirmed in our specimens (Figs. 1, B and C, and 3, A to F and J, and fig. S1). A large medial levator capitis major and a small lateral levator capitis minor have palmately ar-

ranged muscle fibers radiating from the area of origin. Lateral to the articulation of the neck joint (Figs. 1, B and C; 2A; and 3J), an oblique muscle that originates on the inner face of the dermal shoulder girdle and inserts in a hollow on the inner face of the skull roof is identified as the cucullaris muscle (see supplementary text and fig. S1F). It represents the phylogenetically deepest record of this characteristic gnathostome muscle. In mammals, the cucullaris reaches the dorsal midline of the neck, whereas in sharks it is narrower and bounded dorsally by somitic epaxial muscles. The latter morphology is often viewed as primitive for gnathostomes (11), but both differ from the placoderm condition in forming part of a flexible neck region rather than operating a constrained pivoting joint.



The cranial musculature is less well preserved. However, the levator arcus palatini (11) is attached to the ventral postocular process of *Compagopiscis* (Fig. 3, K and L, fig. S2G, and movie S1). In addition, part of the dorsal branchial constrictor muscle can be seen on the visceral surface of the paranuchal plate, lateral to the cucullaris (fig. S1J).

Normal segmented body muscles with antero-posteriorly oriented fibers are present under the dorsolateral parts of the trunk armor and in the tail. However, the posterior part of the ventral abdominal musculature contains two anteroposteriorly

elongated muscle bands with transversely oriented fibers (Figs. 1, C and D, and 2, C to F; see supplementary information), representing areas of stronger fiber development within a continuum of transverse fibers extending across the ventral midline. Dorsolaterally, the bands are in contact with the ventral ends of the myomeres. The existence of these transverse muscles had not been predicted (9).

Mineralized tendinous attachments for the somitic muscles, inclined anterodorsally in a manner reflecting the W shape of the myocommata, are present in the median septum above the neural spines (Fig. 2B). In the eubranchyothoracids,

the myocommata are incompletely preserved (Fig. 3, D to I, and fig. S2F), but in *Austroptyctodus*, they have a shallow W-shaped curvature (12) (fig. S2, H and I). The medial myoseptal attachment probably spanned only two segments, in contrast to at least three segments in modern chondrichthyans and teleosts (13). In extant gnathostomes, the connections between myosepta and skin are biomechanically important (13, 14); preserved *Incisoscutum* skin contains obliquely oriented tendons similar to those in sharks (fig. S2, C to E), suggesting a similar role for the skin as a store of elastic energy (13, 14).

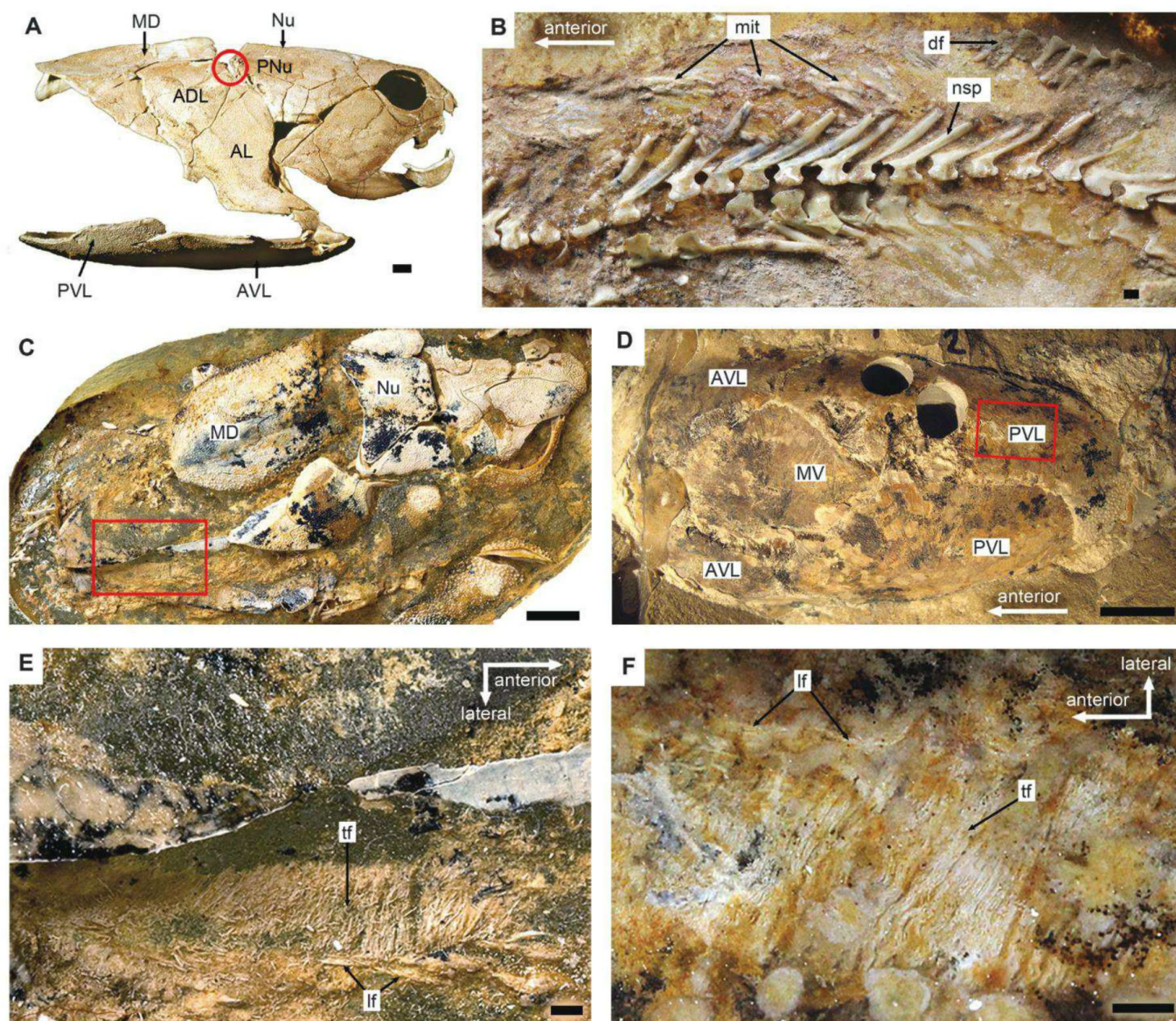


Fig. 2. Dermal skeleton, mineralized tendons, and transverse abdominal muscles. (A) *Eastmanosteus* [Australian Museum (AMF) specimen 82185] lateral view showing the dermal neck joint (indicated by the red ring). (B) *Incisoscutum* [Western Australian Museum (WAM) specimen 03.3.28] mineralized tendinous attachments. (C) *Incisoscutum* [Natural History Museum (NHM) specimen P57636A] dorsal view. The area outlined by the red box is enlarged in (E). (D) *Incisoscutum* (WAM 03.3.24) in ventral view showing body cavity infill. The area outlined by the red box is enlarged in (F). (E)

Transverse and longitudinal abdominal muscles in internal view (NHM P57636A); (F) corresponding muscles in external view (WAM 03.3.24). Scale bars: (A) to (D), 10 mm; (E) and (F), 1 mm. ADL, anterior dorsolateral plate; AL, anterior lateral plate; AVL, anterior ventrolateral plate; df, dorsal fin; lf, longitudinal muscle fibers; MD, median dorsal plate; MV, median ventral plate; mit, mineralized tendons; nsp, neural spine; Nu, nuchal plate; PNu, paranuchal plate; PVL, posterior ventrolateral plate; tf, transverse muscle fibers.

The musculature of eubrachythoracid arthrodires, characterized by shallow myoseptal curvature, differentiated head elevators positioned as antagonists to the cucullaris, and bands of transverse-

fiber muscles in the abdomen, differs greatly from that of sharks. The latter is dominated by deeply W-shaped myomeres, lacks transverse abdominal muscles, and includes a broadly flexible neck

region with a narrow triangular cucullaris forming a dorsal edge to the branchial chamber (11, 13). Most of the differences appear to reflect the overall contrast between a relatively uniform, flexible

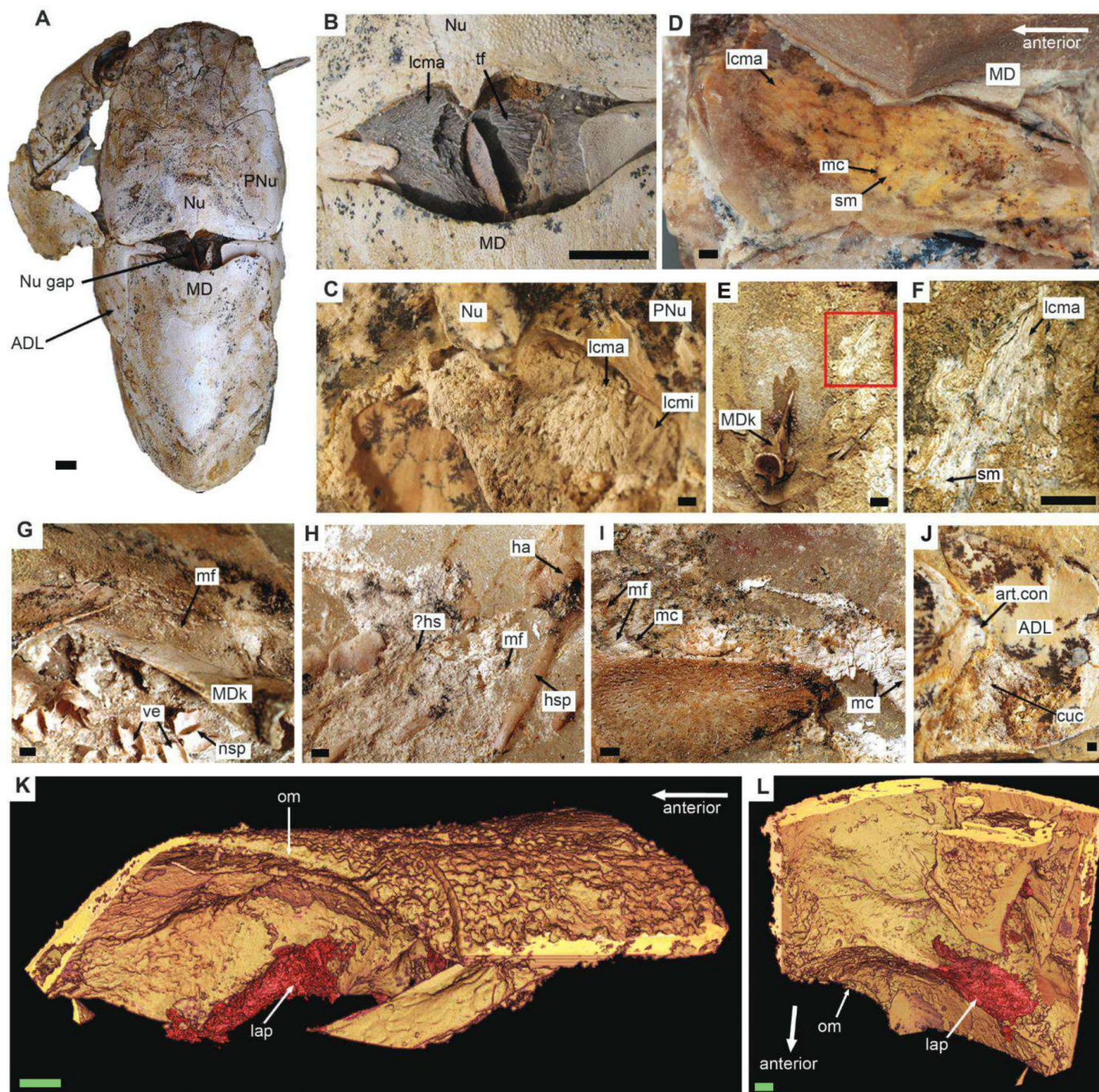


Fig. 3. Muscle preservation. (A) *Eastmanosteus* [Australian National University (ANU) specimen V2582] in dorsal view showing the nuchal gap. (B) Detailed view of ANU V2582. (C) *Compagopiscis* (WAM 11.12.05) in dorsal view, showing the levator capitis major and minor. (D) Dorsolateral view of *Incisoscutum* (WAM 10.01.02) levator capitis major and segmented trunk muscles underlying the median dorsal plate. (E and F) *Incisoscutum* (WAM 95.1.1); internal view of median dorsal plate with muscle fibers. (F) Magnification of red box in (E). (G to I) *Incisoscutum* (NHM P50934). (G) Vertebral elements below the median dorsal plate. (H) Proposed horizontal myoseptum. (I) Trunk muscles. (J) Cucullaris muscle of *Compagopiscis* (WAM

11.12.05), attached to the internal surface of the anterior dorsolateral plate. (K and L) *Compagopiscis* (WAM 12.8.1), showing levator arcus palatini attaching to the left ventral postocular process. (K) Lateral view; (L) posteroventral view. Scale bars: (A) and (B), 10 mm; (C) to (L), 1 mm. Abbreviations are as in Fig. 2, plus: art. con, articular condyle of neck joint; cuc, cucullaris; ?hs, possible horizontal septum; ha, hemal arch; hsp, hemal spine; lap, levator arcus palatini; lcma, levator capitis major; lcmi, levator capitis minor; mc, myocomma; MDk, median dorsal keel; mf, muscle fibers; Nu gap, nuchal gap; om, orbital margin; sm, segmented muscle block; ve, vertebral elements.

body in sharks and a regionalized body with a pivoting neck joint and rigid trunk armor in arthrodires. Their evolutionary importance hinges on whether eubranchyothoracid musculature is specialized or primitive relative to that of sharks. Placoderms appear to be a paraphyletic segment of the gnathostome stem group (3, 4), so if any components of eubranchyothoracid musculature can be shown to be general for placoderms, they can also be inferred to be primitive relative to the crown group. The status of the shallow myoseptal curvature cannot yet be determined in this regard, but the muscles of the neck joint and abdomen have specific skeletal associations that allow such phylogenetic inferences to be drawn.

Most ostracoderms, a grade of jawless stem gnathostomes (2) (Fig. 1A), have head shields that also encompass the shoulder-girdle region (2). This suggests that the gnathostome shoulder girdle originated through subdivision of the shield. Almost all placoderms have a mobile joint between the skull and shoulder girdle, implying the need for elevator and depressor muscles such as those observed in eubranchyothoracids. Thus, a cucullaris operating this joint, antagonistic to specialized epaxial head elevators, is probably primitive relative to the crown gnathostome condition of a cucullaris without specialized antagonists that forms part of a broadly flexible neck.

The transverse abdominal muscles of eubranchyothoracids are not as directly tied to a skeletal structure with an identifiable mechanical function. Comparison with those of a recent elephant shark indicates that these muscles are not homologous with any muscles of the pelvic fin or male clasper (supplementary text). However, the transverse abdominals may modulate shear forces between the armor and the laterally undulating body during tail-propelled swimming. A long ventral armor is also present in antiarchs, recovered as the most primitive placoderms in several recent analyses (3, 4, 15). Transverse abdominal muscles may thus be an attribute of the placoderm segment of the gnathostome stem group and, hence, primitive relative to the absence of such muscles at the base of the gnathostome crown group.

Outside of placoderms, transversely oriented abdominal muscle fibers are restricted to tetrapods and have been regarded as a tetrapod autapomorphy (16). Their associated connective tissues and tendons are derived from the somatopleure component of the lateral plate mesoderm (17), which plays an important role in hypaxial myogenesis (18). In lampreys, the posterior lateral plate mesoderm is not separated into splanchnic and somatopleuric components (19), meaning that it cannot give rise to somatopleure-derived structures such as paired fins. The presence of paired fins in placoderms shows that separation of somatopleure and splanchnopleure had occurred, supporting the inference that their transverse muscles may have been patterned by

the same somatopleure-based mechanism as in tetrapods.

The arthrodires of the Gogo Formation reveal an elaborate regionalized musculature, including the earliest and phylogenetically deepest examples of several muscle types. Particularly surprising is the extensive development of transverse-fiber muscles in the ventral body wall, which parallels the condition in tetrapods. Hypothetical reconstructions are not able to recover the full complexity of this musculature, either on the basis of biomechanical analysis or phylogenetic bracketing, and are thus liable to give a false picture of muscular evolution at the origin of gnathostomes. The study of exceptionally preserved fossils will continue to provide essential data for the reconstruction of vertebrate soft anatomy, particularly in groups with no close living relatives.

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Acknowledgments: We acknowledge M. Siversson at the Western Australian Museum, Perth, and Z. Johanson at the Natural History Museum, London, for lending us specimens in their care. We thank I. Montero Verdú for his picture of the muscle bundles (Fig. 3D) and A. Ritchie for an *Eastmanosteus* image. K.T., P.E.A., and C.B. are supported by Australian Research Council (ARC) QEII Fellowship DP 110101127; J.L., K.T., T.S., and G.Y. by ARC DP 1092870; S.S., V.D., and P.E.A. by European Research Council Advanced Investigator Grant 233111; P.E.A. by a Wallenberg Scholarship from the Knut and Alice Wallenberg Foundation; and C.B. by a Human Frontiers Research Program and an ARC Discovery Project, DP 1096002. The scan performed at the European Synchrotron Radiation Facility in Grenoble, France, was part of project EC770. K.T. acknowledges the 2010 Prime Minister's Science Prize, and J.L. acknowledges funding from The Australian Geographic Society, which supported fieldwork at the Gogo Formation. Specimens are housed in the collections of the Western Australian Museum, Australian National University, Australian Museum, and Museum Victoria, Australia, and the Natural History Museum, UK.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1237275/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S4
References (20–30)
Movie S1

4 March 2013; accepted 29 May 2013
Published online 13 June 2013;
10.1126/science.1237275

Enhanced Remote Earthquake Triggering at Fluid-Injection Sites in the Midwestern United States

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A recent dramatic increase in seismicity in the midwestern United States may be related to increases in deep wastewater injection. Here, we demonstrate that areas with suspected anthropogenic earthquakes are also more susceptible to earthquake-triggering from natural transient stresses generated by the seismic waves of large remote earthquakes. Enhanced triggering susceptibility suggests the presence of critically loaded faults and potentially high fluid pressures. Sensitivity to remote triggering is most clearly seen in sites with a long delay between the start of injection and the onset of seismicity and in regions that went on to host moderate magnitude earthquakes within 6 to 20 months. Triggering in induced seismic zones could therefore be an indicator that fluid injection has brought the fault system to a critical state.

Earthquakes can be induced by underground fluid injection, which increases pore pressure and allows faults to slide under pre-existing shear stress (1). The increase in wastewater

disposal from natural gas development and other sources has been accompanied by an increase in fluid-induced earthquakes in recent years (2). These earthquakes include widely felt earthquakes in

Oklahoma, Arkansas, Ohio, Texas, and Colorado (Fig. 1) (3–7). Although most injection wells are not associated with large earthquakes, the converse is not true. At least half of the 4.5 moment magnitude (M_w) or larger earthquakes to strike the interior of the United States in the past decade have occurred in regions of potential injection-induced seismicity (table S1). In some cases, the onset of seismicity follows injection by only days or weeks (1, 3, 5), and the association with pumping at particular wells is clear. In others, seismicity increases only after months or years of active injection (4, 8, 9).

A long delay before seismic activation implies that faults may be moving toward a critical state for years before failure. However, currently there are no reliable methods to determine whether a particular field has reached a critical state other than by simply observing a large increase in seismicity. This lack of diagnostics is a key problem in developing operational strategies to mitigate anthropogenic activity (2).

Because induced seismic zones are brought to failure by increased pore pressures, we examined whether areas of induced seismicity show a high susceptibility to dynamic triggering by the small transient stresses carried by seismic waves from distant earthquakes. Dynamic triggering in natural settings has been linked to the presence of subsurface fluids, and seismicity rate changes have been shown to depend systematically on the perturbation stress (10–13). This suggests that dynamic triggering could serve as a probe of the state of stress in areas of wastewater injection. We refer to earthquakes that are promoted by anthropogenic activity as induced and to earthquakes that are initiated by transient natural stresses as triggered. By this definition, there can be triggered induced earthquakes.

A search of the Advanced National Seismic System (ANSS) earthquake catalog gives preliminary evidence that induced seismic zones are sensitive to dynamic triggering by surface waves (Fig. 1). Regions of suspected induced seismicity showed a pronounced increase in 3.0 M and larger earthquakes spanning at least a 3-day window after large ($M_w \geq 8.6$) remote earthquakes: the 27 February 2010 8.8 M_w Maule, Chile; 11 March 2011 9.1 M_w Tohoku-oki; and 12 April 2012 8.6 M_w Sumatra earthquakes. The broader central United States shows essentially no response to these events (Fig. 1). Most of the triggering is at three sites: Prague, Oklahoma; Snyder, Texas; and Trinidad, Colorado. Suggestively, each of these regions went on to host mod-

erate to large earthquakes (4.3 to 5.7 M_w) within 6 to 20 months of the strong triggering.

Although the triggering is significant at the 96% level (table S2), a closer investigation is warranted. We therefore enhanced the catalog by applying a single-station matched filter to continuous waveforms (14). The matched-filter approach identifies small, uncataloged earthquakes based on their similarity to target events (15–17). Distinct families of earthquakes are distinguished based on the difference in P and S wave travel times ($S-P$ time), which gives the approximate radial distance from the seismic station (15).

The Cogdell oil field (8), located near Snyder, Texas, hosted a seismic swarm in September 2011 that included a 4.3 M_w main shock (supplementary text). The enhanced catalog shows that the Tohoku-oki earthquake triggered a significant number of earthquakes (14) at this site (Fig. 2 and table S2). In fact, the rate of earthquakes within the 10 days after the Tohoku-oki earthquake was the highest observed over the entire study duration (February 2009 to present), excluding the days immediately after the 4.3 M_w main shock. The triggered earthquakes show a swarm like signature, typical of fluid-induced earthquakes (18), with the largest of the triggered events (3.8 M_w , ANSS) occurring after 2.5 days of smaller events (Fig. 2C). The much earlier February 2010 Maule earthquake did not trigger at Snyder, nor did the post-swarm April 2012 Sumatra earthquake.

Prague, Oklahoma, experienced three 5.0 M_w and greater earthquakes in November 2011, associated with fluid disposal in the Wilzetta field (supplementary text) (4). The enhanced catalog shows that the February 2010 Maule event triggered a strong sequence of earthquakes near the eventual epicenter of the first 5.0 M_w earthquake (Fig. 3 and table S2). The rate of earthquakes in the several days after the Maule trigger far

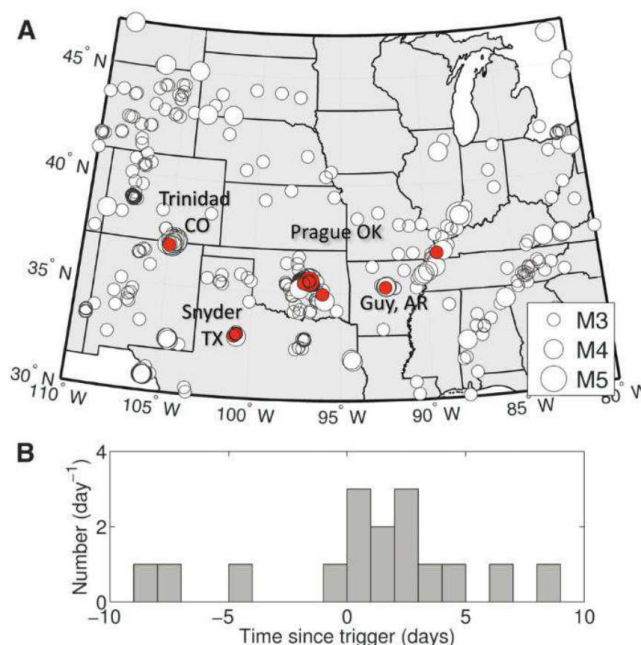
exceeds that of any other time within the period of observation, up to the $M_w \geq 5.0$ earthquakes themselves, which is similar to the observation at Snyder. There are no events detected within ± 32 km relative distance for at least 4 months before the 2010 Maule earthquake.

The largest event in the remotely triggered sequence is a 4.1 M_w , 16 hours after the 2010 Maule earthquake, which may account for the large number of earthquakes that continued up to the time of the first 5 M_w Prague earthquake in 2011 (Fig. 3). If the 4.1 M_w earthquake can be considered a foreshock of the subsequent 5.7 M_w Prague earthquake, then the 5.7 M_w event is not only one of the largest earthquakes to be associated with wastewater disposal (2) but also one of the largest earthquakes to be linked indirectly to a remote triggering event (4, 19).

The April 2011 Tohoku-oki earthquake, which occurred during the ongoing sequence before the 5.7 M_w Prague main shock, did not trigger additional earthquakes near the swarm (Fig. 3 and table S2). The 2012 Sumatra earthquake, on the other hand, followed the main 5.7 M_w Prague earthquake by 5 months and triggered a small uptick in activity that was consistent with the far northeastern tip of the swarm (Fig. 3C). However, this triggered rate change is much smaller than that triggered by the Maule earthquake in 2010.

Trinidad, Colorado, experienced a seismic swarm in August 2011 that included a 5.3 M_w main shock, possibly related to coal-bed methane extraction and reinjection of the produced water in the Raton Basin (supplementary text). The February 2010 Maule earthquake triggered a small but statistically significant response near the site of the 5.3 M_w main shock (Fig. 4 and table S2). Although the total number of triggered events is small (four), the binomial probability of observ-

Fig. 1. Remote triggering in the midwestern United States, from the composite ANSS catalog. (A) Cataloged earthquakes above 3.0 M between 2003 and 2013 (ANSS). Earthquakes in red occurred during the first 10 days after the February 2010, Maule; March 2011, Tohoku-oki; or April 2012, Sumatra earthquakes. Triggering occurs almost exclusively in three injection fields, labeled Prague, Trinidad, and Snyder. **(B)** Stacked earthquake counts in the 10 days before and after the three ≥ 8.6 M_w remote earthquakes. The histogram excludes the Guy, Arkansas, swarm, which dominates event rates at the time of the 2011 Tohoku-oki earthquake but did not trigger (supplementary text).



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Fig. 2. Matched-filter enhanced catalog for Snyder, Texas. (A) Detected events, showing triggering by the 2011 Tohoku-oki earthquake. Symbols along top show strength of triggering (red, strong; green, none). Red star marks 11 September 2011 4.3 M_w main shock (NEIC catalog). Colors correspond to station in (B), with ANSS catalog in gray. Seismometer operating times and the times at which we have enhanced the catalog are shown by thin and thick horizontal bars, respectively. (B) Mapped distances to detected events. Small circles are ANSS catalog earthquakes; a red star shows the main shock. Yellow squares are nearby active injection wells. (C) Cumulative event count around the 2010 Maule and 2011 Tohoku-oki earthquakes.

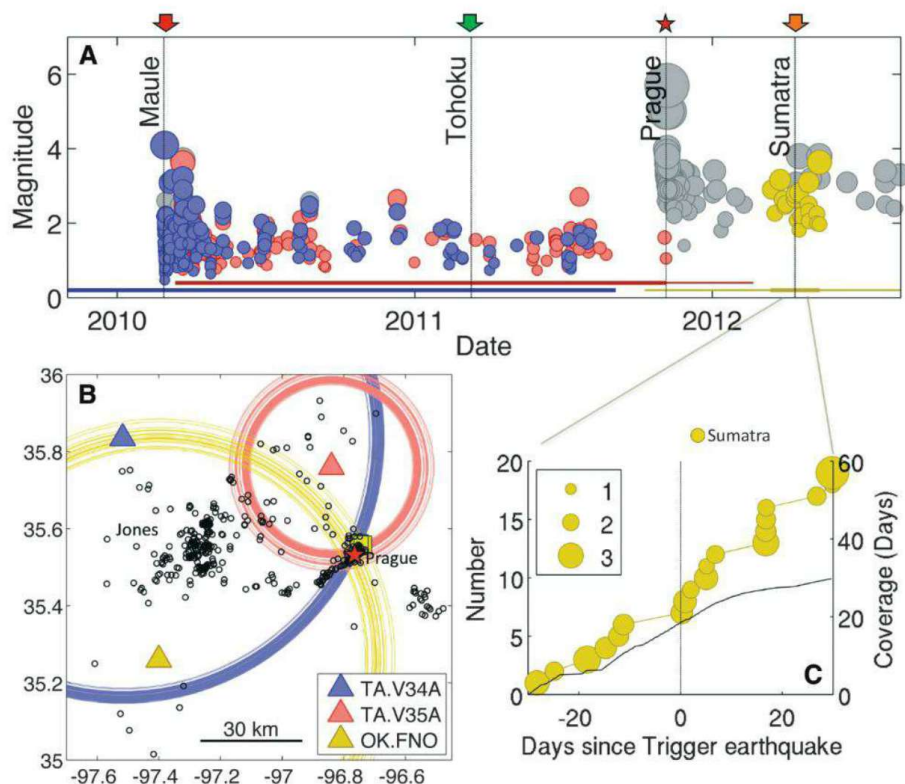
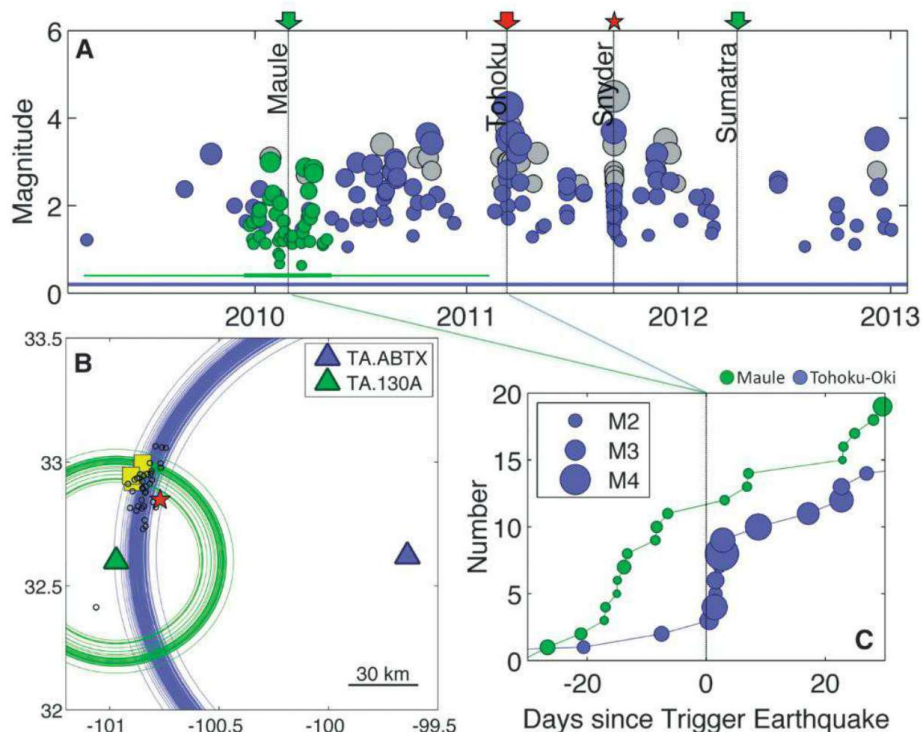


Fig. 3. Matched-filter enhanced catalog for Prague, Oklahoma. (A) Detected events, showing triggering by the 2010 Maule earthquake. Red star marks the 6 November 2011 5.7 M_w main shock. Other details are as in Fig. 2A. (B) Mapped distances to detected events. Details are as in Fig. 2B. (C) Cumulative event count around the 2012 Sumatra earthquake. Cumulative recording time for this intermittently operating station is shown over the same period.

ing this many events in 1 day after the trigger, given five events in the entire previous year, is less than 10^{-5} .

The March 2011 Tohoku-oki earthquake, which occurred during the active portion of the swarm, did not trigger additional seismicity at Trinidad. The

2012 Sumatra earthquake occurred 8 months after the 5.3 M_w Trinidad main shock and triggered a moderate surge in activity that was consistent with the far edge of the swarm, where previous swarm activity had not occurred (fig. S2). This pattern—strong triggering by the first remote earthquake, none by the second, and marginal triggering after the swarm—is very similar to that observed in Oklahoma.

We examined several other regions in the United States that have experienced moderate magnitude earthquakes or heightened seismicity rates linked to fluid injection, including Guy, Arkansas; Jones, Oklahoma; and Youngstown, Ohio. None of these other regions appear to have responded to remote triggering (supplementary text).

The strongly triggered regions were exceptional in that they had a long history of pumping within 10 km of the eventual swarms yet were relatively quiet for much of that history. At other sites of induced moderate earthquakes (Guy, Arkansas, and Youngstown, Ohio), the lag time between the start of pumping and onset of seismicity was as little as months or weeks, presenting a relatively small window of vulnerability to dynamic triggering before the swarms.

The delay in induced seismicity in some regions could be due to complexities in the local geology (supplementary text). In Oklahoma, injection occurred into a fault-bounded pocket, and pressures may have built up slowly over time because of the size of the reservoir bounded by impermeable faults (4). The Cogdell field may have similar isolated pockets, formed by discrete carbonate reefs buried within impermeable shales (8).

Fluids have been suggested as an important component in dynamic triggering since early

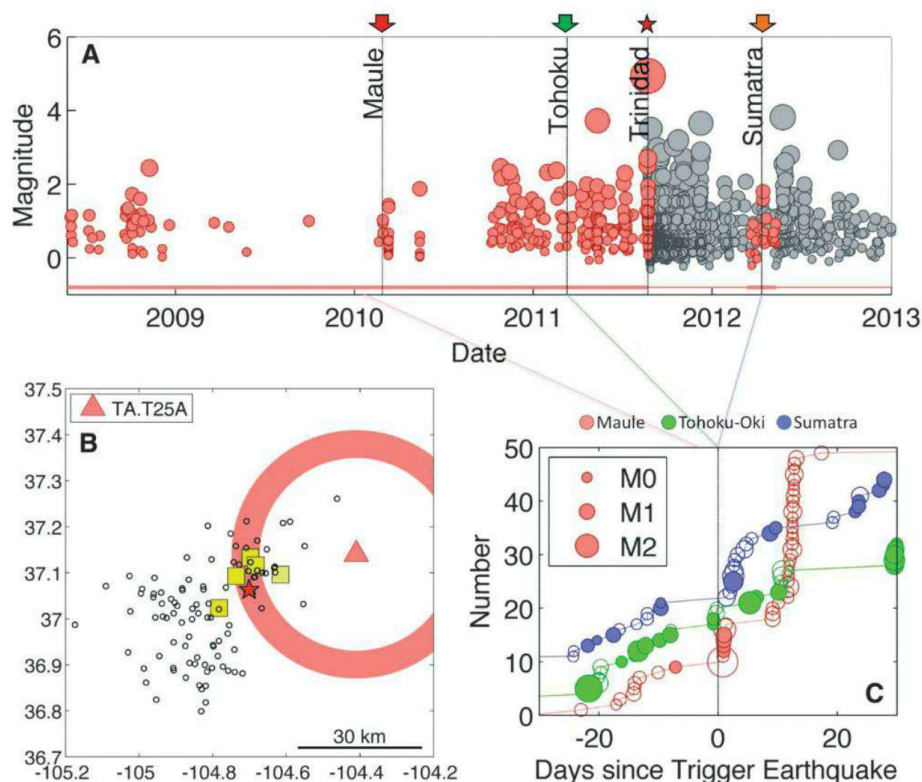


Fig. 4. Matched-filter enhanced catalog for Trinidad, Colorado. (A) Detected events, showing triggering by the 2010 Maule earthquake. Red star marks the 23 August 2011 5.3 M_w main shock. Other details are as in Fig. 2A. (B) Mapped distances to detected events. Details are as in Fig. 2B. (C) Waveform detection counts around the 2010 Maule, 2011 Tohoku-oki, and 2012 Sumatra earthquakes (curves offset for clarity). Filled circles are within 2.5-km radial distance relative to the 5.3 M_w main shock, and open circles are within ~5 km (fig. S2).

observations showed preferential triggering in active volcanic and hydrothermal systems (13, 20, 21). Some features of our observations are also suggestive of a fluid mechanism for triggering. First, in all of the studied cases the triggered earthquakes occurred with a small delay with respect to the passage of the seismic waves, initiating within less than 24 hours and continuing for days to months afterward. This pattern suggests a triggering mechanism that relies on dynamic permeability enhancement and transport of fluids (22, 23), as has been suggested for natural triggered seismicity (20–22). In this scenario, stress transients alter the permeability of hydraulic conduits in the reservoir, accelerating diffusion of pore pressure into local faults. Fractures in active injection reservoirs may be particularly susceptible to this mechanism because the injection of unequilibrated fluids may lead to clogging through mineralization and sedimentation. A brief pressure transient may then flush out these clogged fractures (22, 24).

In Prague and Trinidad, only the first of two large remote events caused earthquakes, despite imparting dilational and shear strains that are similar to subsequent events (table S4). This is also consistent with the permeability enhancement model, which requires a certain amount of recharge time between triggering episodes (24). After local fault slip is triggered, the local permeability rises dra-

matically because of microfracturing and dilation (25), promoting further fluid diffusion over several rupture dimensions (26). Hence, once the seismic swarm is underway the fractures may not return to a state in which they are susceptible to unclogging by small transient stresses.

We find that certain areas of fluid injection are sensitive to small changes in stress associated with the passage of seismic waves from remote large earthquakes. The observations suggest several requirements for an induced region to be sensitive to remote triggering. First, all of the triggered sites in this study had a long history of regional subsurface injection over a period of decades. Second, each triggered site was near to hosting a moderate magnitude earthquake, suggesting critically stressed faults. Last, each site had relatively low levels of seismicity rate in the immediate vicinity (10 km) before the first triggering episode. Remote triggering can therefore indicate that conditions within an injection field have crossed some critical threshold, and a larger induced earthquake could be possible or even likely. This underlines the importance of improved seismic monitoring in areas of subsurface fluid injection.

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Acknowledgments: This paper benefitted from discussions with E. Brodsky and W.-Y. Kim. Injection data for Cogdell Oilfield was provided by C. Frohlich. The Oklahoma Corporation Commission, the Texas Railroad Commission, and the Colorado Oil and Gas Conservation Commission supplied well databases. Earthquake locations were provided by ANSS. Seismic waveforms are from the Incorporated Research Institutions for Seismology Data Management Center. N.J.v.d.E. was supported by U.S. National Science Foundation (NSF) grant EAR-1144503. H.M.S. and G.A.A. were partially supported by U.S. Geological Survey (USGS) National Earthquake Hazards Reduction Program (NEHRP) grant G13AP00024. K.M.K. received support from USGS NEHRP grant G13AP00025. This project made use of EarthScope’s Transportable Array, a facility funded by NSF. The enhanced seismicity catalogs are available as supplementary materials on Science Online.

Supplementary Materials

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Supplementary Text

Figs. S1 to S5

Tables S1 to S5

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Database S1

9 April 2013; accepted 23 May 2013
10.1126/science.1238948

Structural Basis for the Counter-Transport Mechanism of a H^+/Ca^{2+} Exchanger

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Ca^{2+} /cation antiporters catalyze the exchange of Ca^{2+} with various cations across biological membranes to regulate cytosolic calcium levels. The recently reported structure of a prokaryotic Na^+/Ca^{2+} exchanger (NCX_Mj) revealed its overall architecture in an outward-facing state. Here, we report the crystal structure of a H^+/Ca^{2+} exchanger from *Archaeoglobus fulgidus* (CAX_Af) in the two representatives of the inward-facing conformation at 2.3 Å resolution. The structures suggested Ca^{2+} or H^+ binds to the cation-binding site mutually exclusively. Structural comparison of CAX_Af with NCX_Mj revealed that the first and sixth transmembrane helices alternately create hydrophilic cavities on the intra- and extracellular sides. The structures and functional analyses provide insight into the mechanism of how the inward- to outward-facing state transition is triggered by the Ca^{2+} and H^+ binding.

Calcium ions are involved in diverse physiological processes, such as muscle contraction, cell proliferation, exocytosis, and apoptosis (1–3). The Ca^{2+} /cation antiporter (CaCA) superfamily members are important regulators of cytosolic Ca^{2+} levels (4–6). They use the electrochemical gradient of other cations—such as Na^+ , H^+ , or K^+ —to catalyze Ca^{2+} transport across biological membranes (4–6). The CaCA superfamily comprises five major groups: Na^+/Ca^{2+} exchangers (NCX), K^+ -dependent Na^+/Ca^{2+} exchangers (NCKX), H^+/Ca^{2+} exchangers (CAX), cation/ Ca^{2+} (other cation than Na^+ , H^+ , or K^+) exchangers (CCX), and the bacterial homologous gene (*YrbG*) (6, 7). All CaCA proteins contain two highly conserved α -repeat regions (α -1 and α -2), which are thought to have arisen from an ancient gene duplication event (6, 8) and are reportedly important for cation binding and transport (8–10).

Recently, the crystal structure of the Na^+/Ca^{2+} exchanger from *Methanococcus jannaschii* (NCX_Mj) in an outward-facing state was reported, revealing a pseudosymmetric architecture formed by two structural repeats of five transmembrane (TM) helices with opposite orientations (10). Three Na^+ sites and one Ca^{2+} -specific site were observed within the cation binding pocket, leading to an exchange model in which the sequential binding of three Na^+ ions causes

the release of Ca^{2+} during the exchange cycle (10). However, the mechanism by which Ca^{2+} and the counter-transported cations stimulate the structural transition between the inward- and outward-facing states remains elusive (4, 5, 11) because of the lack of the structural information of its inward-facing state. Furthermore, the molecular basis for the cation recognition in other members of the CaCA superfamily remains unclear.

We performed the structural analysis of a CaCA homolog from *Archaeoglobus fulgidus* (CAX_Af). Liposome- and *Escherichia coli*-based transport assays confirmed that the homolog has H^+/Ca^{2+} exchange activity (Fig. 1A and figs. S1 to S3). The purified CAX_Af protein was crystallized by the lipidic cubic phase (LCP) method (12) under low-pH (6.0 to 6.5) conditions. The structure was determined with the multiple anomalous diffraction method by using mercury derivatives and refined to 2.3 Å resolution (table S1). The crystallographic asymmetric unit contained two molecules (mol A and mol B) (fig. S4). Because the overall architectures of these two molecules were essentially identical (root mean square deviation of 1.04 Å over all Ca atoms), we focused on the mol A structure.

The structure of CAX_Af contains 12 TM helices, with both N and C ends located on the intracellular side (Fig. 1, B to D). The core domain (TM2 to TM5 and TM7 to TM10) is tightly packed together, whereas the gating bundle, consisting of the long TM1 and TM6 helices, is loosely packed against the core domain (Fig. 1, C and D). The core domain shares structural similarity with NCX_Mj (10); their N and C terminal halves (TM2 to TM5 and TM7 to TM10, respectively) are related by a pseudo twofold rotational axis within the molecule. The two conserved α -repeats (6) consist of TM2 and TM3 for α -1 and TM7 and TM8 for α -2 (figs. S5 and S6). CAX_Af contains two additional TM helices

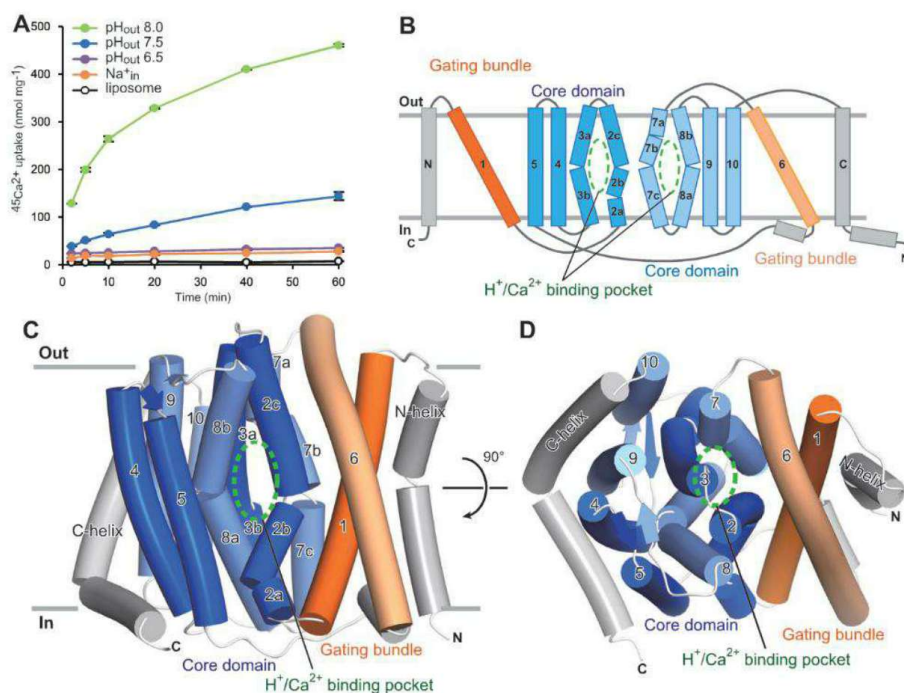


Fig. 1. The overall structure and function of CAX_Af. (A) Time courses of $^{45}Ca^{2+}$ uptake by liposome-reconstituted CAX_Af at different pH values. (B) Schematic representation of the CAX_Af topology. The core domain and the gating bundle are colored blue and orange, respectively. The additional helices are gray. The H^+/Ca^{2+} binding pockets are indicated by green dotted circles. (C and D) Structure of CAX_Af (mol A) as viewed from (C) the membrane plane or (D) the extracellular side. The color coding is the same as in (B).

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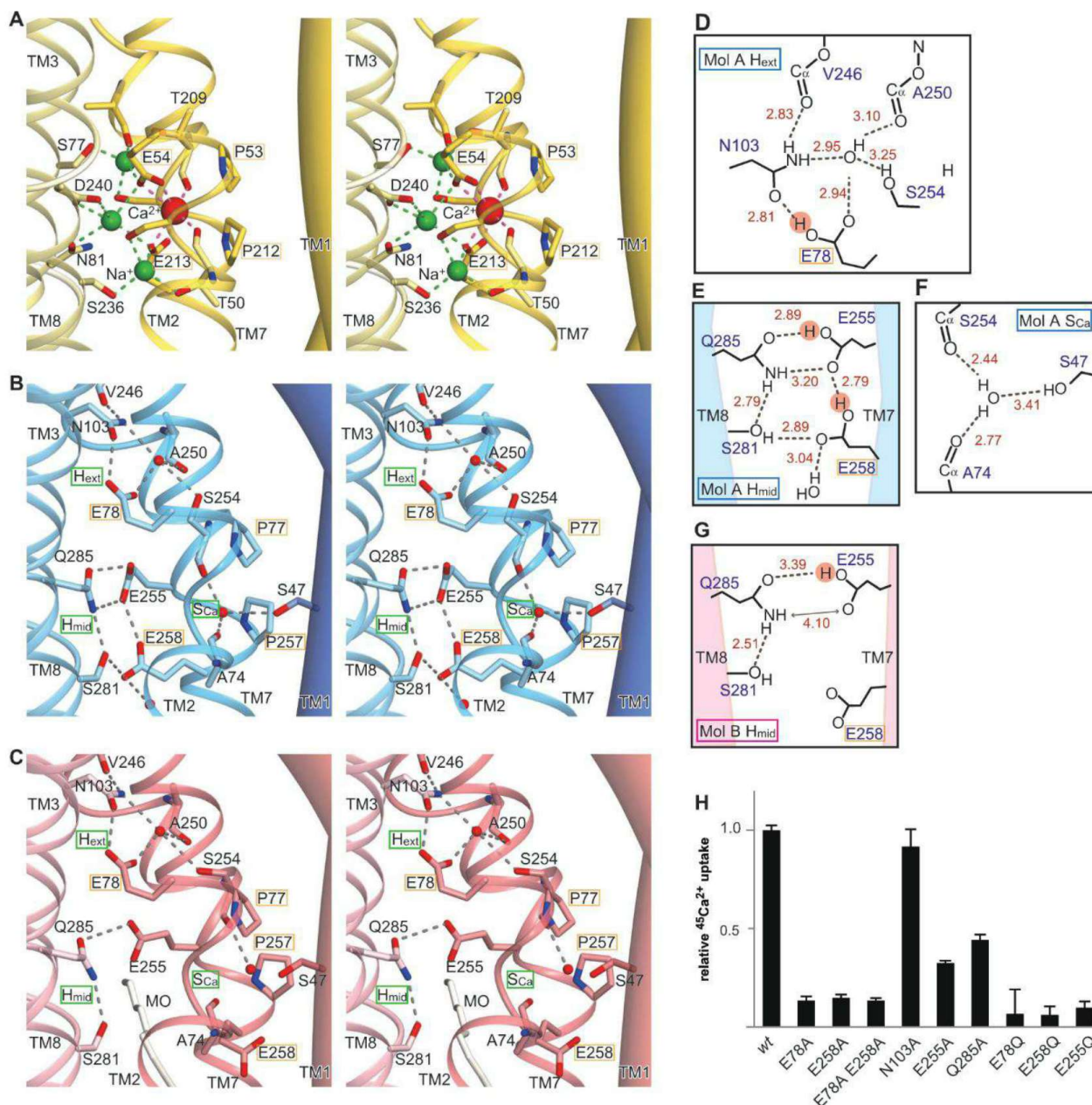


Fig. 3. Cation binding pocket. (A to C) Stereo views of (A) the $\text{Na}^+/\text{Ca}^{2+}$ binding pocket in NCX_Mj and [(B), mol A; (C), mol B] the $\text{H}^+/\text{Ca}^{2+}$ binding pocket in CAX_Af. The signature motifs in the α -repeats are highlighted by orange rectangles. In (A), the bound Na^+ and Ca^{2+} ions are depicted with green and red spheres, respectively. In (B) and (C), coordinated water mol-

ecules are shown as red spheres. The hydrogen-bond networks, H_{ext} and H_{mid} , and the putative Ca^{2+} binding site, S_{Ca} , are indicated. (D to G) Schematic drawings of the hydrogen bond geometry in the $\text{H}^+/\text{Ca}^{2+}$ binding pocket [(D) to (F), mol A; (G) mol B]. (H) Liposome-based $^{45}\text{Ca}^{2+}$ uptake assays of mutants.

crystal was obtained under low-pH conditions (pH 6.0 to 6.5). The Ala or Gln mutation of either Glu⁷⁸, Glu²⁵⁵, or Glu²⁵⁸ decreased the $\text{H}^+/\text{Ca}^{2+}$ exchange activity (Fig. 3H), indicating the importance of their protonation and deprotonation during the exchange cycle. The residues involved in the H_{ext} and H_{mid} networks (Ser²⁵⁴, Glu²⁵⁵, Ser²⁸¹, and Gln²⁸⁵) are located at equivalent positions to the Na^+ coordinating residues in NCX_Mj (Fig. 3, A and B, and fig. S5). Taken together, the structural comparison with NCX_Mj suggests that the space formed between the kinks of TM2 and TM7 is the Ca^{2+} binding site (S_{Ca})

(Fig. 3B), whereas the hydrogen bonding networks (H_{mid} and H_{ext}) (Fig. 3B) involving Glu⁷⁸, Glu²⁵⁵, and Glu²⁵⁸ can function as the H^+ binding sites.

The Glu⁷⁸ and Glu²⁵⁸ residues are involved in both Ca^{2+} and H^+ binding, further suggesting that the binding of Ca^{2+} and H^+ to these sites is mutually exclusive, which is consistent with the counter-transporting mechanism of the CaCA superfamily (4). The present crystal structure presumably represents the H^+ bound state and thus cannot accommodate Ca^{2+} . Supporting this notion, Ca^{2+} is not bound to S_{Ca} , even though

the crystal was obtained in the presence of 10 mM CaCl_2 (fig. S7).

As described above, the crystallographic asymmetric unit contains two molecules of CAX_Af (mol A and mol B). The mol B structure exhibited different hydrogen bonding patterns in the H_{mid} network. In mol B, the carboxylate group of Glu²⁵⁸ does not form hydrogen bonds with Ser²⁸¹ and Glu²⁵⁵ and is exposed toward the inward-facing cavity (Fig. 3C), suggesting that Glu²⁵⁸ is deprotonated (Fig. 3G and fig. S8). As a result, H_{mid} is partially disrupted in mol B (Fig. 3, C and G). In mol A, the hydrogen bond

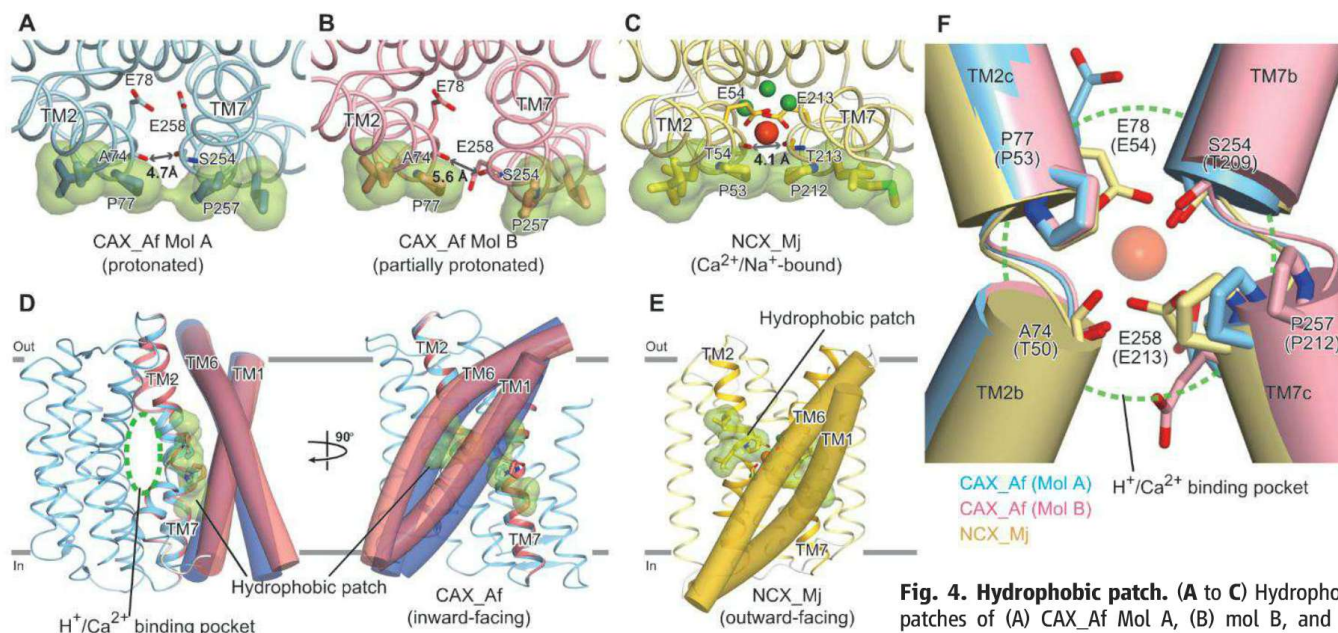


Fig. 4. Hydrophobic patch. (A to C) Hydrophobic patches of (A) CAX_Af Mol A, (B) mol B, and (C) NCX_Mj are shown with green surface-rendered CPK

models. (D and E) Structural comparison of (D) CAX_Af (mol A in blue and mol B in magenta) and (E) NCX_Mj. The hydrophobic patches and gating bundles are highlighted. (F) Structural comparison of TM2 and TM7 in CAX_Af mol A (blue), mol B (magenta), and NCX_Mj (yellow). The residues of NCX_Mj are represented in parentheses.

interactions in H_{mid} bridge TM7 and TM8, stabilizing the arrangement of TM7 in the core domain (Fig. 3, B and E). In contrast, in mol B the fewer hydrogen bonds in H_{mid} resulted in the slight twisting of TM7 as compared with that in mol A (Fig. 3C and fig. S10), allowing the accommodation of a monoolein acyl chain in the inward-facing cavity. This twisting of TM7 changes the directions of its side chains; Pro²⁵⁷ is shifted toward the gating bundle (fig. S10). Consequently, there is a large gap between Pro⁷⁷ (TM2) and Pro²⁵⁷ (TM7) in mol B (Fig. 4B). Altogether, these observations suggested that mol B represents a “partially” protonated state, whereas mol A represents the “fully” protonated state after binding H^+ . The protonation of Glu²⁵⁸ in H_{mid} tightens the interaction between TM7 and TM8, which results in the twisting of TM7 toward the H^+/Ca^{2+} binding pocket to close the gap between Pro⁷⁷ and Pro²⁵⁷ (Fig. 4, A and B, and fig. S10).

In the structure of NCX_Mj, Pro⁵³ and Pro²¹² at the kinks of TM2 and TM7 and their neighboring hydrophobic residues, Leu⁵² and Ile⁵⁵ (TM2) and Leu²¹¹ and Leu²¹⁴ (TM7), form a flat hydrophobic patch at the center of the interface between the core domain and the gating bundle (Fig. 4, C and E), which probably enables the sliding motion of the gating bundle, which is important for the transition between the inward- and outward-facing states (10). In mol A of CAX_Af, the corresponding hydrophobic patch is formed by Leu⁷⁶, Pro⁷⁷, and Ala⁸⁰ (TM2) and Ala²⁵³, Pro²⁵⁷, and Ile²⁶⁰ (TM7) (Fig. 4A). In contrast, in mol B the hydrophobic patch is split by the gap between Pro⁷⁷ and Pro²⁵⁷ (Fig. 4B). Thus, the H^+ binding to CAX_Af closes the gap in the hydrophobic patch and enables the gating bundle to slide to the outward-facing state. Consistent with

this mechanism, the gating bundle in mol A is slightly shifted toward the outward-facing state (Fig. 4D).

A structural comparison of the Ca^{2+} binding sites between CAX_Af and NCX_Mj suggested that Ca^{2+} binding induced conformational changes in TM2 and TM7. In the structure of NCX_Mj, the distance between the backbone carbonyls at the kinks of TM2 and TM7 (Thr⁵⁰ and Thr²⁰⁹) (Fig. 4, C and F) that coordinate Ca^{2+} is 4.1 Å (Fig. 4C). In contrast, in mol B of CAX_Af the distance between the corresponding carbonyl oxygen atoms is 5.6 Å (Fig. 4B). Consequently, TM2 and TM7 of CAX_Af are twisted toward the gating bundle, as compared with those of NCX_Mj (Fig. 4F and fig. S11). The twisting of TM7 is more substantial than that of TM2 (fig. S11B). The structural comparison suggests that Ca^{2+} binding to CAX_Af induces the twisting of TM2 and TM7 toward the H^+/Ca^{2+} binding pocket (Fig. 4F). This twisting changes the directions of the backbone carbonyls at the kink of TM2 and TM7 and also the side chains of Glu⁷⁸ and Glu²⁵⁸, enabling optimal coordination geometry for the Ca^{2+} as observed in NCX_Mj (Fig. 4F and fig. S11B). Thus, similar to the case of H^+ binding, Ca^{2+} binding brings Pro⁷⁷ and Pro²⁵⁷ close together (Fig. 4F and fig. S11). Last, this closing the gap enables the sliding of the gating bundle.

The crystal structure of CAX_Af revealed the inward-open conformation of the CaCA protein, which revealed the alternate formation mechanism of the hydrophilic cavities on the intracellular and extracellular sides. With the concomitant functional analyses, the present structural analysis suggested how the binding of either Ca^{2+} or H^+ induces the structural change (fig. S12): The gap between the TM2 and TM7 fixes the gating bun-

dle in the inward-facing apo structure to prevent ion leakage across the membrane (4), whereas the binding of either Ca^{2+} or H^+ induces the gap closure, which enables the sliding motion of the gating bundle, as predicted from the NCX_Mj structure (10). Further discussion of the structural change mechanism may require the elucidation of the CAX_Af structure in the Ca^{2+} bound state.

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Acknowledgments: We thank H. Nishimasu and M. Hattori for useful discussions and critical comments on the manuscript; T. Tsukazaki for sharing beamtime and useful discussion; A. Kurabayashi for technical assistance; the beam-line staffs at BL32XU and BL41XU of SPring-8 for assistance in data collection; and the RIKEN BioResource Center (Ibaraki, Japan) for providing the *Archaeoglobus fulgidus* genomic DNA. The diffraction experiments were performed at SPring-8 BL32XU and BL41XU (proposals 2012A1093, 2012A1201, and 2012A1087) and with the approval of RIKEN. Part of this work was performed with the support of the Radioisotope Center, University of Tokyo. This work was supported by the Japan Society for the Promotion of Science (JSPS) through its “Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST program)” to O.N., by the Core Research for Evolutional Science and Technology (CREST)

Program "The Creation of Basic Medical Technologies to Clarify and Control the Mechanisms Underlying Chronic Inflammation" of Japan Science and Technology Agency (JST) to O.N., by a grant for HPCI STRATEGIC PROGRAM Computational Life Science and Application in Drug Discovery and Medical Development by MEXT to R.I., by a Grant-in-Aid for Scientific Research on Innovative Areas (23136517) from Ministry of Education, Culture, Sports, Science and Technology (MEXT) to S.K., by a Grant-in-Aid for Scientific Research (C) (23590319) from JSPS to T.I., and by a Grant-in-Aid for Scientific Research (S) (24227004) and a Grant-in-Aid for Young Scientists (A) (22687007) from the MEXT to O.N. and

R.I., respectively. T.N. expressed and purified CAX_Af for crystallization, collected the diffraction data, solved the structures, and performed functional analyses in liposomes. N.F. screened CaCA genes and identified CAX_Af. S.K. and T.I. performed transport assays in *E. coli* cells. A.D.M. performed transport assays in *E. coli* spheroplasts. G.K. made mutants. K.H. assisted with data collection. S.O. supported crystallization. N.D. analyzed the purified protein by mass spectrometry. T.N., R.I., and O.N. wrote the manuscript. R.I. and O.N. directed and supervised all of the research. The coordinates and the structure factors have been deposited in the Protein Data Bank (PDB) under accession codes 4KPP.

Supplementary Materials

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10 April 2013; accepted 15 May 2013
Published online 23 May 2013;
10.1126/science.1239002

Crystal Structure of NLRC4 Reveals Its Autoinhibition Mechanism

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Nucleotide-binding and oligomerization domain–like receptor (NLR) proteins oligomerize into multiprotein complexes termed inflammasomes when activated. Their autoinhibition mechanism remains poorly defined. Here, we report the crystal structure of mouse NLRC4 in a closed form. The adenosine diphosphate–mediated interaction between the central nucleotide-binding domain (NBD) and the winged-helix domain (WHD) was critical for stabilizing the closed conformation of NLRC4. The helical domain HD2 repressively contacted a conserved and functionally important α -helix of the NBD. The C-terminal leucine-rich repeat (LRR) domain is positioned to sterically occlude one side of the NBD domain and consequently sequester NLRC4 in a monomeric state. Disruption of ADP-mediated NBD-WHD or NBD-HD2/NBD-LRR interactions resulted in constitutive activation of NLRC4. Together, our data reveal the NBD-organized cooperative autoinhibition mechanism of NLRC4 and provide insight into its activation.

Nucleotide-binding and oligomerization domain (NOD)–like receptors (NLRs) constitute a crucial component of the cytosolic immunosurveillance system of mammals by detecting the signature components of pathogens and consequently triggering immune responses (1–4). Dysregulation of NLR function has been associated with a variety of diseases (5–7).

NLR proteins typically comprise a varied N-terminal effector domain, such as caspase-recruitment domain (CARD) or pyrin domain; a central NOD; and a C-terminal leucine-rich repeat (LRR) domain (1). NLRs belong to the signal transduction adenosine triphosphatases (ATPases) with numerous domains (STAND) subfamily, including Apaf-1 and CED-4 (8). The current model of NLR activation posits that ligand binding to the C-terminal LRR sensor domain results in exchange of adenosine diphosphate (ADP) for ATP followed by oligomerization (8). Indeed, ligand-induced oligomerization was shown for several NLR members, including NLRC4 (NLR

family CARD domain–containing protein 4) (9–14). Oligomerization results in the recruitment of signaling molecules, which together with NLRs,

make up the inflammasome, a multiprotein complex that triggers host innate immune responses and rapid cell death. The NLRC4 inflammasome is activated in mice by bacterial flagellin (14–19) or the components of type 3 secretion systems (14, 15, 19, 20).

In order to understand the structure of NLRC4, we made a mouse NLRC4 (mNLRC4) mutant with the CARD (residues 1 to 89) and the internal residues (622 to 644) deleted (mNLRC4 Δ CARD) (fig. S1). mNLRC4 with the latter deletion only was still functional (fig. S2). Similar to the full-length mNLRC4 protein and consistent with previous data (14), the mNLRC4 Δ CARD protein showed no defect in protein folding (fig. S3) and was monomeric in solution (fig. S4). The crystal structure of mNLRC4 Δ CARD was solved at a resolution of 3.2 Å (table S1 and fig. S5).

The overall structure of mNLRC4 Δ CARD is shaped like an inverted question mark (Fig. 1). The NOD module comprises the nucleotide-binding domain (NBD), the helical domain HD1, and the winged-helix domain (WHD). As observed in all

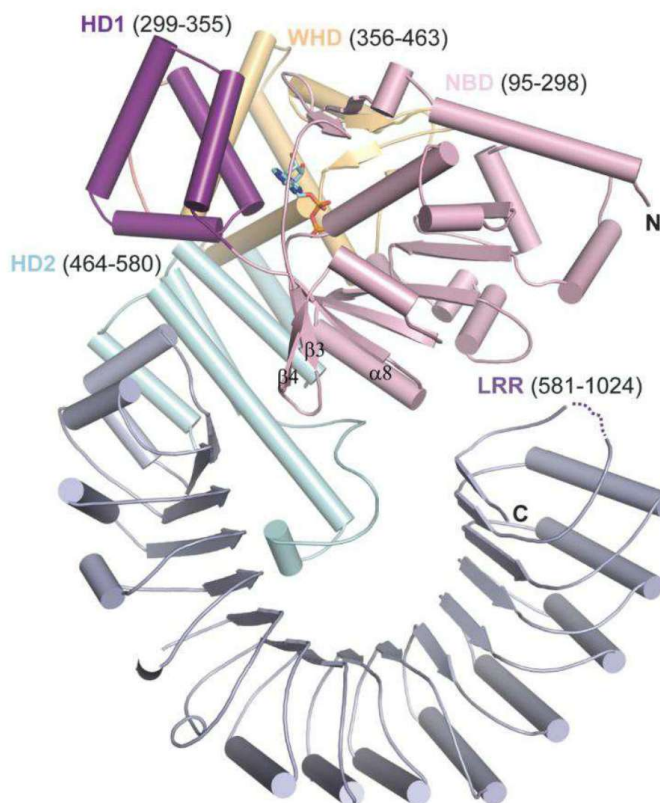


Fig. 1. Overall structure of mNLRC4 Δ CARD. The overall structure of mNLRC4 Δ CARD shown in cartoon. The structural domains of mNLRC4 Δ CARD are labeled, and the numbers following their labels indicate their boundaries. The bound ADP molecule is shown in stick and cyan. Some of the structural elements are labeled. The dashed line indicates the disordered region (residues 1011 to 1014). "N" and "C" represent N terminus and C terminus, respectively.

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the other AAA+ ATPase structures (21), the NBD of mNLRC4 Δ CARD is a three-layered α/β structure but possesses an additional β hairpin ($\beta 3$ and $\beta 4$) (Fig. 1). In vitro study showed that the

mNLRC4 protein was an active ATPase (fig. S6). Although not supplemented during the experiment, an ADP molecule was well defined by the electron density (fig. S7). The HD2 domain caps

the N-terminal side of the LRR domain via extensive interactions (fig. S8). The LRR domain is located distant from the HD1 and WHD domains and the ADP-binding site (Fig. 1). Its structural

Fig. 2. Critical role of the ADP-mediated WHD-NBD interaction in mNLRC4 autoinhibition.

(A) The ADP-bound mNLRC4 Δ CARD is in a closed conformation. Structural comparison of the NOD of mNLRC4 Δ CARD with that of the inactive Apaf-1 (PDB code 3SFZ). Color codes for domains of mNLRC4 Δ CARD and Apaf-1 are indicated. The bound ADPs (in stick) in mNLRC4 Δ CARD and in Apaf-1 are shown in cyan and yellow, respectively. (B) Recognition of ADP by mNLRC4. Detailed interactions of ADP with mNLRC4 from the area highlighted in (A). The dashed red lines indicate polar (hydrogen and salt) interactions. (C) Transfection of 293T cells with plasmids as indicated. A twofold serial dilution was made for the transfection of H443L. After 24 hours, the culture medium was supplemented with PA (protective antigen) and LF_N (N-terminal domain of anthrax lethal factor)—FliC (C-terminal part of flagellin) proteins. The cells were lysed, and the cleaved IL-1 β was detected by anti-IL-1 β immunoblotting analysis after 12 hours. β -actin was used as a loading control. The experiments were repeated five times. WT, wild type; HA, hemagglutinin. (D) Transfection of 293T cells with plasmids as indicated. Thirty-six hours after the transfection, cells were lysed and subjected to gel-filtration chromatography. All the fractions were collected and detected by anti-Myc epitope immunoblotting analysis.

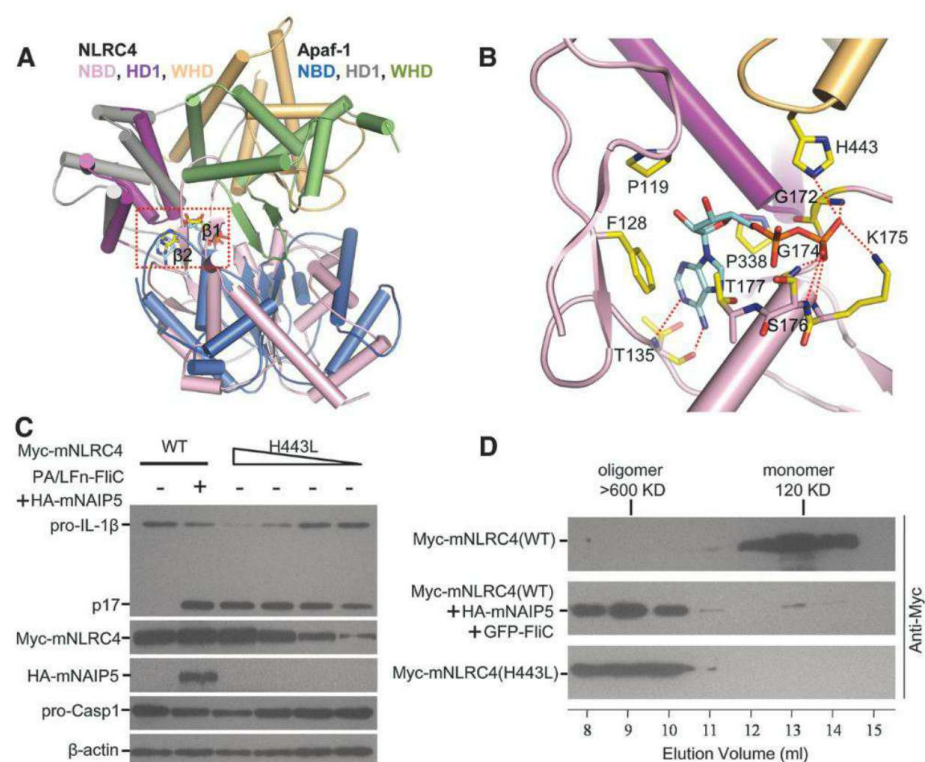
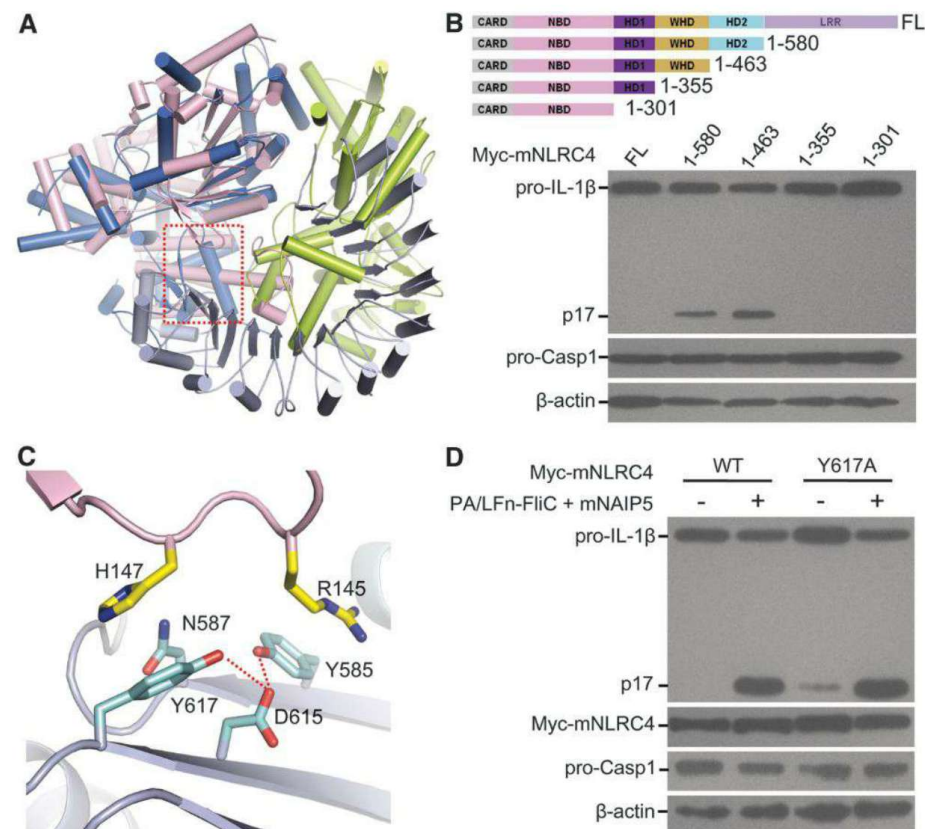


Fig. 3. The C-terminal LRR domain sequesters mNLRC4 in a monomeric state.

(A) The LRR domain of mNLRC4 Δ CARD overlaps with one protomer of CED-4 from a lateral dimer. Shown is the structural superposition of mNLRC4 Δ CARD with a lateral dimer of CED-4 (PDB code 3LQQ). The NOD of mNLRC4 Δ CARD was used as the template to superimpose with one protomer of a CED-4 lateral dimer. The LRR is shown in slate, the remaining part of mNLRC4 Δ CARD in pink, and the two CED-4 protomers in blue and green. (B) (Top) Schematic diagram of mNLRC4 truncation mutants. (Bottom) The assay was performed as described in Fig. 2C, and 293T cells were transfected with plasmids as indicated. The experiment was repeated three times. FL, full length. (C) Detailed LRR-NBD interactions from the area highlighted in (A). The side chains from NBD are shown in yellow and those from LRR in cyan. D, Asp; N, Asn. (D) Analysis of mutants disrupting LRR-NBD interactions. The assay was performed as described in Fig. 2C and repeated three times.



coupling with the NOD is established through the additional β hairpin (Fig. 1). Marginal NBD-LRR interactions results in closure of the solenoid structure.

A DALI search identified the NOD domains of Apaf-1 (22, 23) and CED-4 (24, 25) as the closest homologs to mNLRC4 Δ CARD (Fig. 2A and fig. S9). Compared with the closed form of Apaf-1, mNLRC4 Δ CARD possesses an extra β sheet (β 1 and β 2) that pushes the WHD away from the NBD (Fig. 2A). Nevertheless, the WHD of mNLRC4 Δ CARD still faces the front of the ADP binding site, indicating that the ADP-bound mNLRC4 Δ CARD adopts a closed conformation according to previous classification (8). This is in contrast with the WHD of CED-4 (25) (fig. S9A), although the structures of the three WHDs are well superimposed (fig. S10). ADP appears to be critical for locking mNLRC4 Δ CARD in an inactive conformation. The phosphate groups of ADP structure the Walker A motif (fig. S1), with five hydrogen bonds formed between them (Fig. 2B). Two additional hydrogen bonds between ADP and the NBD come from coordination of the N1 and N6 atoms in the adenine but not the guanine base with Thr¹³⁵. Like His⁴³⁸ in the inactive

Apaf-1, His⁴⁴³ from the WHD of mNLRC4 Δ CARD also hydrogen bonds with the β -phosphate group of the ADP molecule (Fig. 2B).

The WHD of Apaf-1 undergoes notable structural remodeling with respect to its NBD after oligomerization (23, 26). This is also expected for the WHD of mNLRC4, given its similar positioning to inactive Apaf-1 (Fig. 2A). Thus, the interaction of His⁴⁴³ with the β -phosphate group is likely specific for the ADP-bound mNLRC4. Disruption of the interaction would facilitate conformational changes in the WHD and attenuate ADP binding, both of which favor mNLRC4 activation. We, therefore, examined the activity of the mutant His⁴⁴³→Leu⁴⁴³ (H443L) in processing interleukin (IL)-1 β by using the assays established previously (14, 19). The mutant protein, even when expressed at a lower level than that of the wild-type mNLRC4, bypassed the requirement of flagellin and mouse NLR family, apoptosis inhibitory protein 5 (mNAIP5, which functions upstream of NLRC4) for IL-1 β activation (Fig. 2C). Consistent with its constitutive activity, the mutant protein formed a higher order of oligomers when overexpressed in 293T cells (Fig. 2D).

Oligomerization of CED-4 (25), Apaf-1 (27), and the *Drosophila* Apaf-1 (DARK) (27) involves a conserved mode of domain organization: One side of the NBD from one protomer stacks against the opposite side of the NBD from the other protomer in a lateral dimer (fig. S11). Structural superposition of mNLRC4 Δ CARD with one protomer of a lateral CED-4 dimer (24) revealed that the LRR domain completely overlaps with the other CED-4 protomer (Fig. 3A), suggesting that the LRR has a role in sequestering mNLRC4 in a monomeric state. Indeed, the LRR deletion led to a constitutively active mNLRC4 in processing of IL-1 β (Fig. 3B and fig. S12A), consistent with previous data (14). Further removal of the HD2 appeared to result in more-efficient mNLRC4-mediated IL-1 β activation (Fig. 3B and fig. S12B).

Wedging of Tyr⁶¹⁷ between the LRR domain and the NBD appears to be important for the interaction between the two domains (Fig. 3C). It stacks against His¹⁴⁷ from the NBD and forms an Asp⁶¹⁵-mediated hydrogen bond with Tyr⁵⁸⁵, which in turn packs against Arg¹⁴⁵. Supporting the structural observations, the mutant Y617A (Y, Tyr; A, Ala) constitutively activated IL-1 β (Fig. 3D,

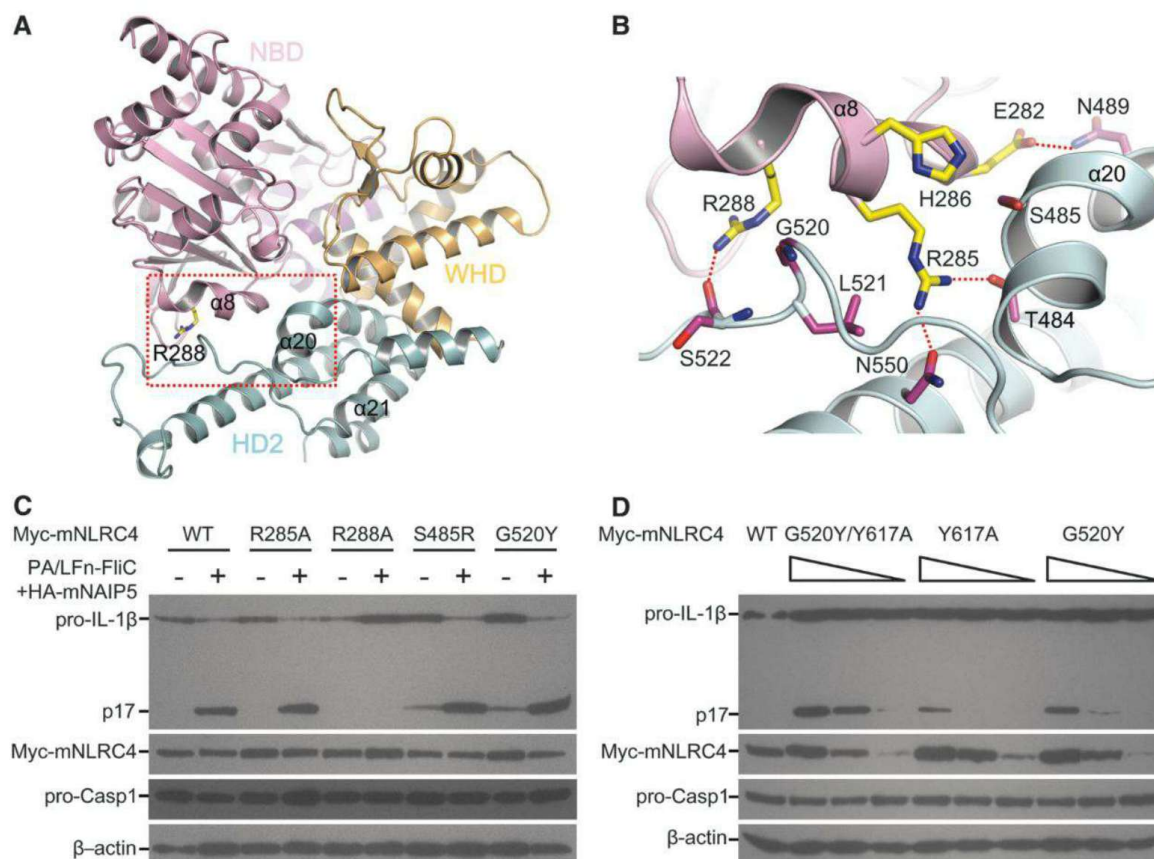


Fig. 4. HD2 negatively regulates the function of a conserved α helix from NBD. (A) The α 8 helix from the NBD is occluded by HD2. Structural elements involved in the HD2-NBD interaction are labeled. (B) Detailed HD2-NBD interactions from the area highlighted in (A). The side chains from the NBD and the HD2 are shown in yellow and pink, respectively. E, Glu; T, Thr. (C) Analysis of mutants with disrupting HD2-NBD

interactions disrupted. The assay was performed as described in Fig. 2C and repeated for three times. (D) Analysis of a mNLRC4 variant with the HD2-NBD and LRR-NBD interactions disrupted. The assay was performed as described in Fig. 2C. A twofold serial dilution was made for the transfection of G520Y/Y617A, G520Y, and Y617A. The experiments were repeated three times.

fig. S13A and fig. S14). Consistent with its partially constitutive activity, the mutant was still responsive to flagellin (Fig. 3D).

HD2 exists in all NLRs (8), but whether and how it contributes to NLR autoinhibition remain unknown. The HD2 of mNLRC4ΔCARD is positioned differently from that of the inactive Apaf-1 (fig. S9) but similarly to the WHD of CED-4 that is involved in the formation of the CED-4 apoptosome (24) (fig. S11A). These structural observations suggest that HD2 may have a role in mNLRC4 autoinhibition. Consistent with this, the mNLRC4 mutant lacking HD2 and LRR domains was more efficient at activating IL-1 β than the mutant lacking the LRR domain only (Fig. 3B). HD2 contacts α 8 from NBD (Fig. 4, A and B), a conserved structural component involved in oligomerization of STAND family members (8, 21, 24). Mutation of Arg²⁸⁸ but not Arg²⁸⁵ of α 8 to alanine abrogated flagellin-induced IL-1 β activation (Fig. 4C and fig. S13C), supporting a critical role for α 8 in mNLRC4 activation. Failure of the R288A (R, Arg) protein to activate IL-1 β was not caused by its defect in folding or in interaction with mNAIP5 (fig. S15).

The NBD-HD2 interaction is mainly mediated by packing of α 8 against α 20 and the loop C-terminal to α 21 (Fig. 4B). Ser⁴⁸⁵ from α 20 and Gly⁵²⁰ from the loop act as supporting points for the packing, which is further strengthened by Arg²⁸⁵ wedged between the loop and α 20. The interactions with the HD2 domain result in steric masking of α 8. As anticipated, the mutants S485R and G520Y (S, Ser; G, Gly) constitutively activated IL-1 β (Fig. 4C, and figs. S13B and S14) but were still responsive to flagellin (Fig. 4C). The variant carrying the mutations of Y617A and G520Y became more efficient for ligand-independent IL-1 β activation than either of the single mutants (Fig. 4D), suggesting a cooperative inhibition of mNLRC4 by the LRR and HD2 domains. Together, our data show that HD2 acts as an autoinhibitory domain by negatively regulating the function of the conserved α 8 in mNLRC4 activation.

The effects generated by the mutation H443L (Fig. 2, C and D) demonstrate the important role of the His⁴⁴³-ADP interaction in NLRC4 autoinhibition. Given the conserved histidine (28) from other NLR proteins, some of the disease-related mutations (2–4) in the NLR proteins are expected to perturb a similar interaction and result in their constitutive activation. The LRR-mediated NLRC4 inhibition is reminiscent of CED-4 inhibition by CED-9 in which CED-9 blocks CED-4 oligomerization (25). In addition, the first WD40 domain in the inactive Apaf-1 overlaps with the LRR domain of mNLRC4 and an adjacent protomer of a lateral dimer from the Apaf-1 apoptosome (26) (fig. S16).

The close locations to a potential ligand-binding site (fig. S17A) make it possible for the HD2-NBD and the LRR-NBD interfaces to be perturbed upon ligand binding. The extensive WHD-HD2 (Fig. 1B) and HD2-LRR interactions (fig. S8) appear not sufficiently labile to be disrupted to allow substantial conformational changes

in one domain with respect to the other two. Additionally, phosphorylation of Ser⁵³³ (pS533) (fig. S18), which is critical for assembly of the mNLRC4 inflammasome (15), acts to stabilize the HD2-LRR interaction. Thus, ligand binding may disengage the three domains as a whole from the NBD, rendering it accessible to a second NLRC4 molecule for oligomerization (fig. S17B). pS533 can have a role in this process through unknown mechanisms. Regardless of the mechanism of intermediates, it seems that ligand binding allosterically activates the assembly of the NLRC4 inflammasome.

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Acknowledgments: We thank P. Schulze-Lefert for critically reading the manuscript, F. Yu and J. He at Shanghai Synchrotron Radiation Facility BL17U1 for data collection, L. Yu for helpful suggestions on culturing 293T cells, G. He from Y. Yan's laboratory assistance with CD, and J. Yang from F. Shao's laboratory for suggestions on cell-based assays. The coordinates and structural factors for mNLRC4ΔCARD have been deposited in the Protein Data Bank (PDB) with the accession code 4KXF. This research was funded by the National Outstanding Young Scholar Science Foundation of China (20101331722) and State Key Program of National Natural Science of China (31130063) to J. Chai.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1236381/DC1
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11 February 2013; accepted 30 May 2013
Published online 13 June 2013;
10.1126/science.1236381

Biosynthesis of Antinutritional Alkaloids in Solanaceous Crops Is Mediated by Clustered Genes

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Steroidal glycoalkaloids (SGAs) such as α -solanine found in solanaceous food plants—as, for example, potato—are antinutritional factors for humans. Comparative coexpression analysis between tomato and potato coupled with chemical profiling revealed an array of 10 genes that partake in SGA biosynthesis. We discovered that six of them exist as a cluster on chromosome 7, whereas an additional two are adjacent in a duplicated genomic region on chromosome 12. Following systematic functional analysis, we suggest a revised SGA biosynthetic pathway starting from cholesterol up to the tetrasaccharide moiety linked to the tomato SGA aglycone. Silencing *GLYCOALKALOID METABOLISM 4* prevented accumulation of SGAs in potato tubers and tomato fruit. This may provide a means for removal of unsafe, antinutritional substances present in these widely used food crops.

Our demand for more and better food continues to increase. Improved nutritional qualities, as well as removal of antinutritional traits, are needed. Various approaches have been used to add nutritional qualities to food crops. We focus here on reducing the level of endogenous, antinutritional factors in existing crops (1). Anti-

nutritional substances range from lethal toxins to compounds that disrupt digestion and nutrient absorption (2). In the course of crop domestication, levels of antinutrients were reduced by selection and/or breeding, although some of such substances remain in the general food source. In addition, wild germplasm, which can be useful as a source of

novel traits such as pathogen resistance, may also be complicated by co-occurrence of antinutritional compounds that need to be removed. Current technologies include extensive backcrossing, which can be a slow and imperfect process (3).

Steroidal glycoalkaloids (SGAs), found in staple vegetable crops such as potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*), are a class of antinutritional substances that remain in our food chain and daily diet (4). The glycoalkaloids α -solanine (5) and α -chaconine are the principal toxic substances in potato. These SGAs cause gastrointestinal and neurological disorders and, at high concentrations, may be lethal to humans. Mechanisms of toxicity include

disruption of membranes and inhibition of acetylcholine esterase activity (6). For this reason, total SGA levels exceeding 200 mg per kg fresh weight of edible tuber are deemed unsafe for human consumption (7). SGA biosynthesis requires genes encoding uridine 5'-diphosphate (UDP)-glycosyltransferases (UGTs) that decorate the steroidal alkaloid (SA) skeleton with various sugar moieties (8, 9). The tomato GLYCOALKALOID METABOLISM 1 (*GAME1*) glycosyltransferase, a homolog of the potato *SGT1* (8), catalyzes galactosylation of the alkaline tomatidine (9). Cholesterol is the proposed common precursor for biosynthesis of both steroidal alkaloids (SAs) and non-nitrogenous steroidal saponins (STs) (Fig. 1 and fig. S1) (10). Conversion of cholesterol to the alkaline SA should require several hydroxylation, oxidation, and transamination reactions (10). Here, we identify genes encoding enzymes performing the conversion of cholesterol to SGAs and use them to engineer *Solanaceae* plants with reduced SGA content.

To discover genes associated with SGA biosynthesis, we carried out coexpression analysis using transcriptome data from tomato and potato plants (11). Sixteen genes from each species were coexpressed with *GAME1/SGT1* (Fig. 2). One of these genes, which we named *GLYCOALKALOID METABOLISM 4 (GAME4)*, encodes a member of

the 88D subfamily of cytochrome P450 proteins (fig. S2). *GAME4* and *GAME1/SGT1* display a very similar expression profile in tomato and potato (fig. S3, B and C, and fig. S4). We then discovered that the *GAME1/SGT1* and *GAME4* genes in tomato and potato are positioned in chromosomes 7 and 12, respectively, such that they are physically next to several of their coexpressed genes (Fig. 3).

A cluster of *GAME1/SGT1* coexpressed genes spans a ~200 kilo-base pair genomic region on chromosome 7. Together with *GAME1*, the tomato cluster is composed of seven coexpressed genes. These include three UDP-glycosyltransferases [*GAME2* (termed *SGT3* in potato), *GAME17*, and *GAME18*], a cytochrome P450 of the 72A subfamily (*GAME6*), a 2-oxoglutarate-dependent dioxygenase (*GAME11*), and a cellulose synthase-like protein. It appears that in potato this cluster contains five coexpressed genes as it lacks homologs of the tomato *GAME17* and *GAME18* UDP-glycosyltransferases. We performed enzyme activity assays with the four recombinant clustered tomato UDP-glycosyltransferases. *GAME17* and *GAME18* exhibited UDP-glucosyltransferase activity when incubated with tomatidine galactoside (T-Gal) and γ -tomatine (T-Gal-Glu) as a substrate, respectively, whereas *GAME2* was shown to have a UDP-xylosyltransferase activity when incubated with β 1-tomatine (T-Gal-Glu-Glu) as a

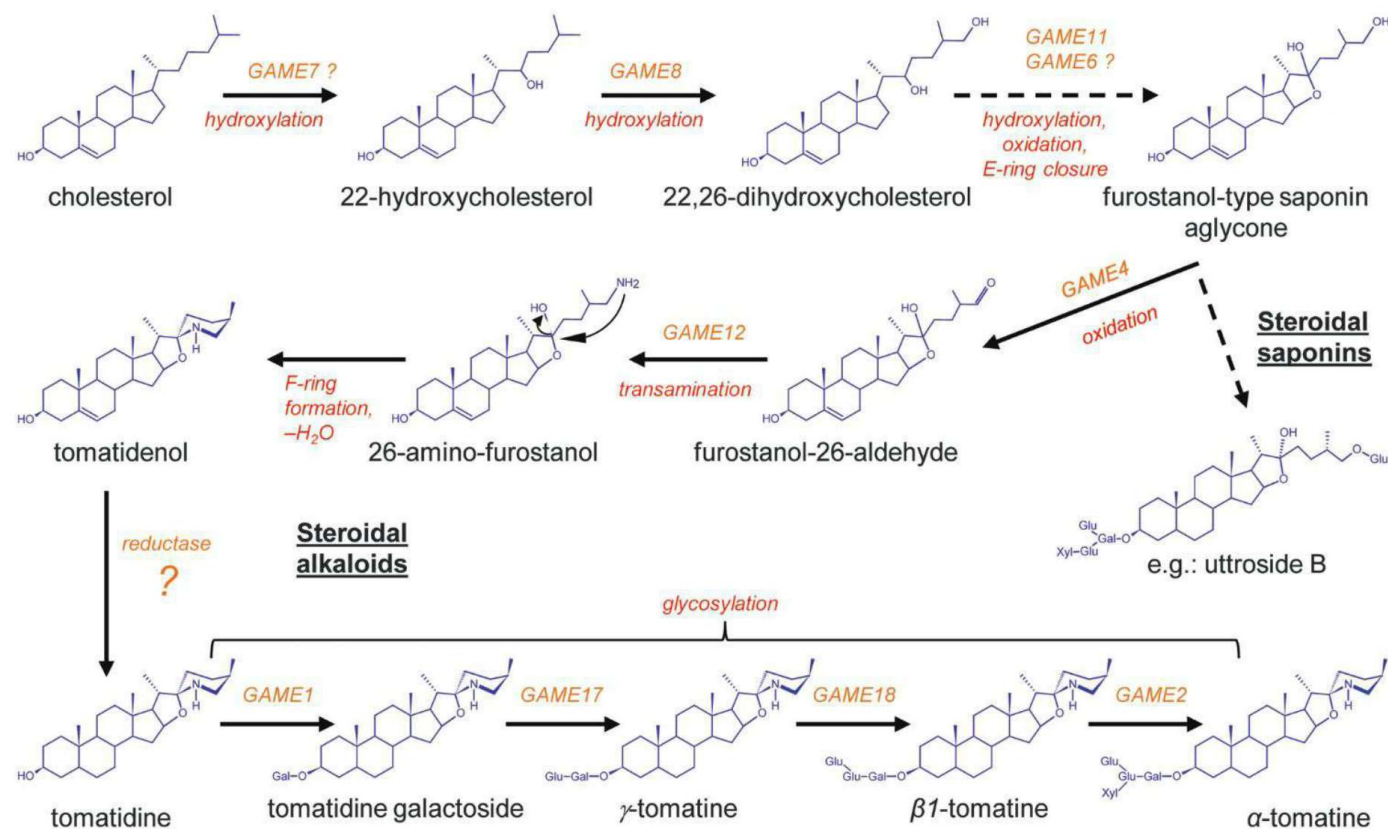


Fig. 1. Biosynthesis of steroidal alkaloids and saponins in the triterpenoid biosynthetic pathway in *Solanaceae* plants. Suggested biosynthetic pathway from cholesterol toward α -tomatine. Dashed and solid arrows represent multiple or single enzymatic reactions in the pathway,

respectively. The proposed activity of *GAME1*, *GAME4*, and *GAME8* was supported by investigating transgenic plants; of *GAME11*, *GAME12*, and *GAME18* by VIGS assays; and of *GAME1*, *GAME17*, *GAME18*, and *GAME2* by activity assays of the recombinant enzymes.

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substrate (Fig. 4, F to H, and fig. S5). GAME1 was previously shown to act as a tomatidine UDP-galactosyltransferase in tomato (9). When incu-

bating the four recombinant UGT enzymes in a single test tube, with tomatidine, and all glycoside donors (UDP-galactose, -glucose and -xylose), we

observed the accumulation of the final SGA product, α -tomatine (Fig. 4I and fig. S5). The role of *GAME18* in creating the tetrasaccharide moiety

Fig. 2. Steroidal alkaloids gene discovery through coexpression network analysis in *Solanaceae* plants. Shared homologs of coexpressed genes for “bait-genes” from tomato (*SIGAME1* and *SIGAME4*) and potato (*StSGT1* and *StGAME4*). Continuous [correlation coefficient (r) > 0.8] and dashed (r > 0.63) lines connect coexpressed genes. *, located in the tomato or potato chromosome 7 cluster. St, *Solanum tuberosum*; Sl, *S. lycopersicum*. Color background of gene names corresponds to the bait with which they were found to be coexpressed (as above). (For more details, see tables S1 to S10.) SP, serine proteinase; PI, proteinase inhibitor; UPL, ubiquitin protein ligase; ELP, extensin-like protein; PK, protein kinase; SR, sterol reductase; RL, receptor-like.

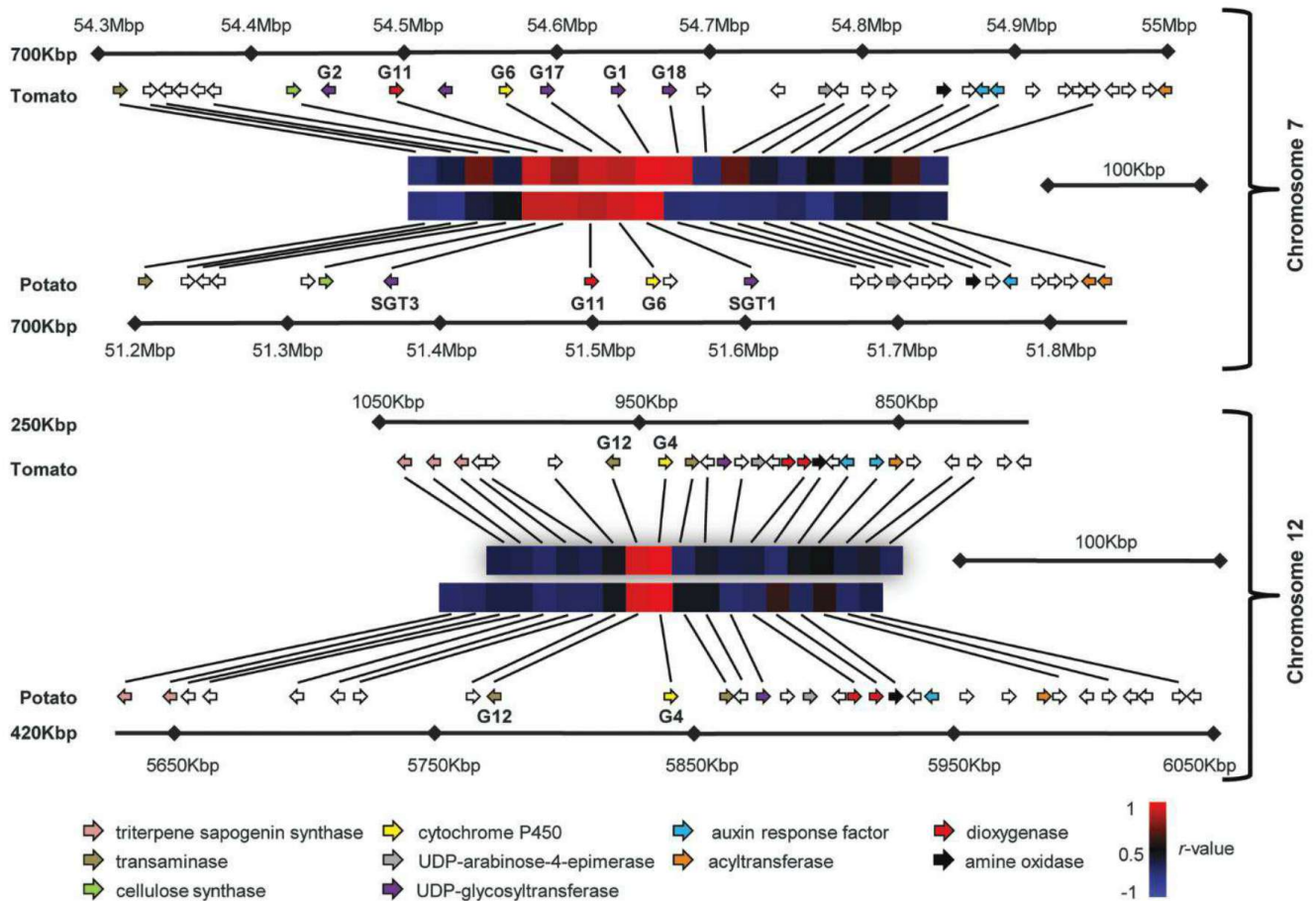
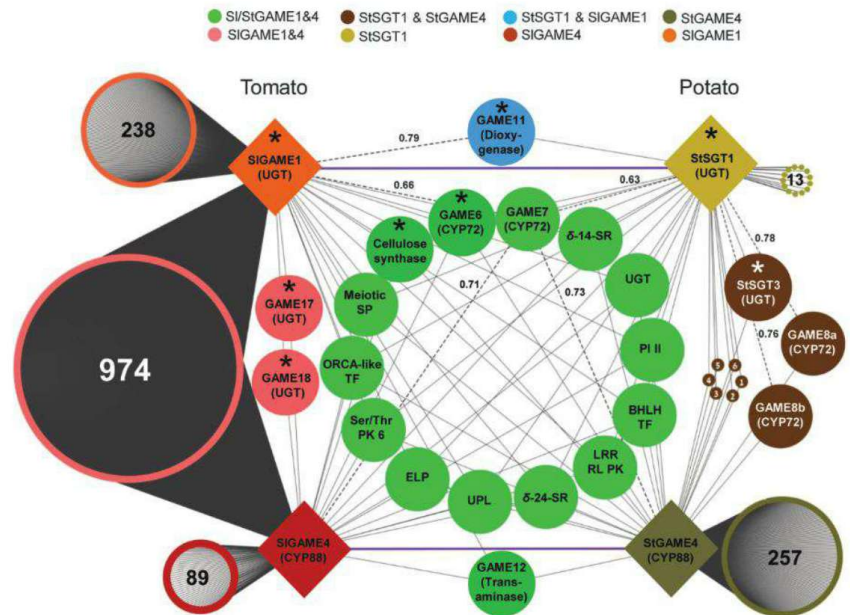


Fig. 3. Schematic map of genes identified in the duplicated genomic regions in tomato and potato and their coexpression. Coexpression with *GAME1/SGT1* (chromosome 7) and *GAME4* (chromosome 12) as baits in either potato or tomato are presented in the form of a heat map (table S12). Specific

gene families are indicated by colored arrows, whereas members of other gene families are shown by white arrows. Note the homology in genes flanking the high-coexpression regions and positioned in a matching sequence along the genome, suggesting a common origin of the regions on both chromosomes (see fig. S11).

of α -tomatine was supported by virus-induced gene silencing (VIGS) assays as *GAME18*-silenced fruit accumulated γ -tomatine that was not present in the control sample (fig. S6, A to E). Analysis of the tomato leaves, silenced (VIGS) in *GAME11*, a putative dioxygenase in the cluster, revealed a significant reduction in α -tomatine levels and accumulation of several cholesterol-type steroidal saponins, confirming its function in the SGA pathway (Fig. 4B and fig. S6, F to I). Additionally, *GAME6*, encoded by another cluster gene, was previously suggested to be associated with SGA metabolism (12).

GAME4 and a putative transaminase (*GAME12*) that was highly coexpressed were positioned in close proximity to each other on chromosome 12 of both species (Fig. 3). Silencing *GAME4* in potato by RNA interference (RNAi) (*GAME4i* plants), showed a reduction by a factor of up to 74 in the levels of α -solanine/chaconine and other SGAs in both leaves and tubers (fig. S7, A to E). In the dark, normal quantities of α -solanine and α -chaconine are 200 and 370 mg/kg, respectively (fig. S7C). After light exposure, levels of α -solanine and α -chaconine increase in tuber skin, and quantities are 510 and

870 mg/kg, respectively. With the *GAME4* gene silenced, the concentrations of both α -solanine and α -chaconine remained below 5 mg/kg and did not change with light exposure (fig. S7, C to E).

In the domesticated tomato, the dominant SGA in leaves and mature green fruit is α -tomatine that was reduced by a factor of ~ 100 in *GAME4i* plants (figs. S7F and S8 and table S14). During the transition from green to red fruit, α -tomatine is converted to lycopersides and esculeosides. These two classes of compounds represent hydroxylated, glycosylated, and often acetylated α -tomatine derivatives (13). Hence, reduced α -tomatine accumulation in the green fruit stage resulted in reduced accumulation of lycopersides and esculeosides in the red-ripe fruit stage (fig. S7G). Complementary results were obtained in *GAME4*-overexpressing tomato plant leaves (*GAME4oe*), as they accumulated 2.5 times as much α -tomatine as the controls (fig. S8B). Furthermore, *GAME4oe* red-ripe fruit exhibited 2.9 times more esculeoside A (fig. S8C), demonstrating once more the central role of *GAME4* in SGA biosynthesis. It appeared that SGA precursors [i.e., cholesterol, cycloartenol,

and (S)-2,3-oxidosqualene] and the phytosterols campesterol and β -sitosterol accumulated in leaves of *GAME4*-silenced tomato plants (fig. S9). Despite altered phytosterol levels, *GAME4*-silenced plants were not affected in their morphology under the conditions examined in this study (14).

Tomato and potato *GAME4i* plants with decreased levels of SGAs accumulated nitrogen-lacking compounds identified as STSs (fig. S7, H and I, and fig. S10). Greater reduction in SGAs correlated with greater accumulation of STSs (fig. S7, D, E, H, and I). Levels of STSs were significantly induced by light in several wild-type and *GAME4i* lines examined (fig. S7, H and I). These results indicate that SGAs and STSs originate from the same precursor and that *GAME4* is positioned in a branch point before the incorporation of nitrogen for SGA generation in the diverging biosynthetic pathways that produce these two classes of steroidal compounds (Fig. 1 and fig. S1).

GAME12 (transaminase)-silenced tomato leaves were found enriched with a furostanol-type saponin (Fig. 4D and fig. S6, J to M), suggesting additional hydroxylation of its accumulated substrate. We also

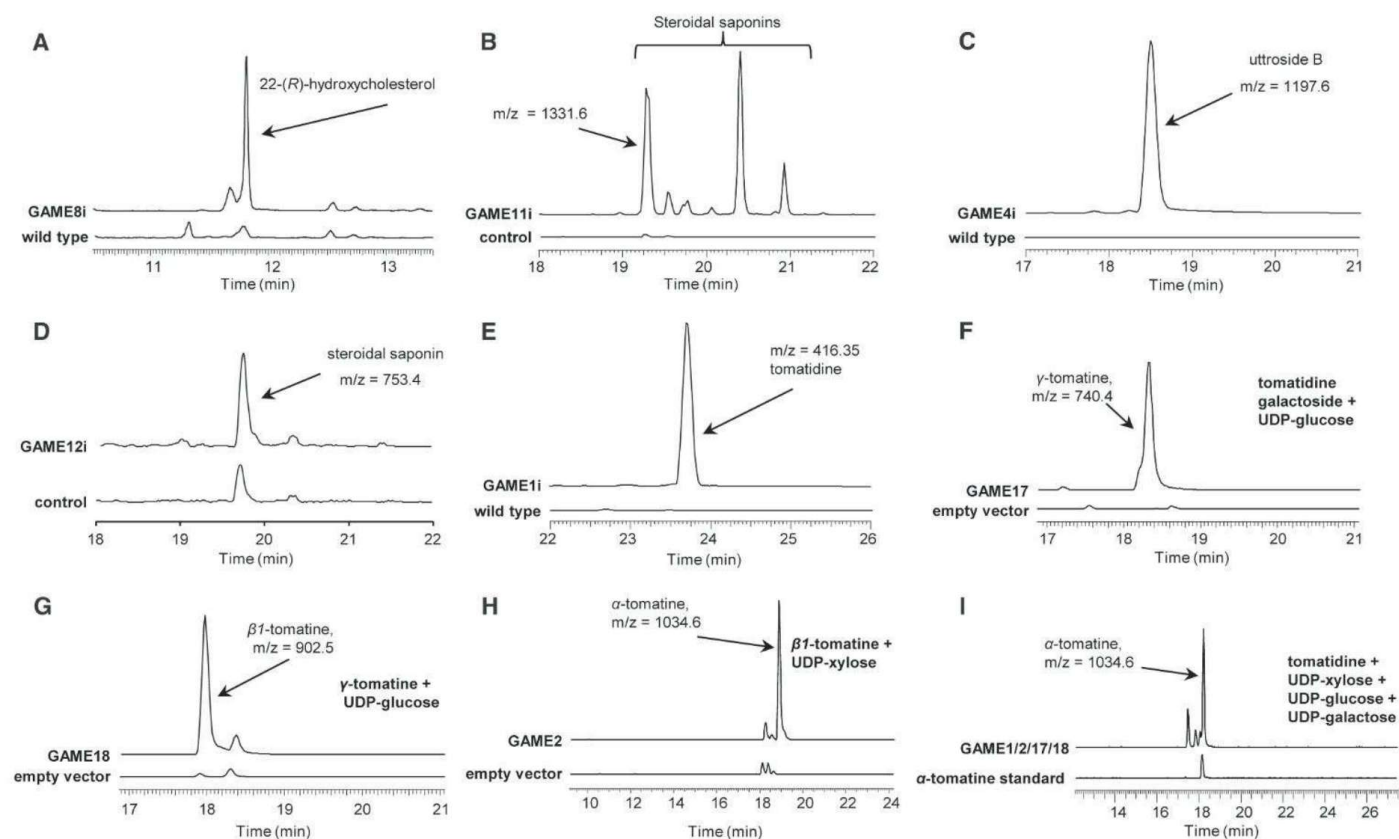


Fig. 4. Functional analysis of tomato *GAME* genes. (A) *GAME8*-silenced transgenic (RNAi) leaves accumulated 22-(R)-hydroxycholesterol compared to wild type. (B) An array of cholesterol-type steroidal saponins (STSs) accumulates in *GAME11* VIGS-silenced leaves. (C) An STS annotated as Uttroside B accumulates in *GAME4*-silenced transgenic leaves. (D) An STS [mass/charge ratio (m/z) = 753.4] accumulates in *GAME12* VIGS-silenced leaves. (E) Tomatidine, the steroidal alkaloid aglycone, accumulates in *GAME1*-silenced transgenic leaves. (F to I) Enzyme activity assays of the four recombinant tomato *GAME* glycosyltransferases (14). Reactions containing *GAME17* (F) and *GAME18* (G)

recombinant proteins with UDP-glucose as donor-substrate, and T-Gal or T-Gal-Glu γ -tomatine as an acceptor-substrate, respectively, produced products with m/z = 740.4 and m/z = 902.5, respectively. Reaction products were identified as γ -tomatine for *GAME17* (F) and T-Gal-Glu-Glu for *GAME18* (G). Reaction containing β 1-tomatine, the *GAME2* recombinant protein, and UDP-xylose produced α -tomatine (H). Reaction containing tomatidine as substrate, UDP -galactose, -glucose and -xylose as sugar donors, and the *GAME1*, *GAME2*, *GAME17*, and *GAME18* recombinant proteins resulted in accumulation of α -tomatine (I). See also figs. S5, S6, and S10.

functionally examined genes that were tightly coexpressed and positioned elsewhere in the genome that belong to the CYP72 subfamily of cytochrome P450s (i.e., *GAME7* and *GAME8*). *GAME7* was coexpressed in both species, whereas *StGAME8a* and *StGAME8b* were strongly coexpressed with *StSGT1* and *StGAME4* in potato. At present, we could not demonstrate SGA-related activity for *GAME7*, although as for *GAME6*, it was suggested to be involved in SGA metabolism (12). Yet, *GAME8*-silenced tomato leaves accumulated 22-(*R*)-hydroxycholesterol (fig. S6, N to Q), a proposed intermediate in the SGA biosynthetic pathway (Fig. 1).

The above findings allowed us to propose a pathway from cholesterol to α -tomatine. Cholesterol is hydroxylated at C22 by *GAME7* (12), followed by *GAME8* hydroxylation at the C26 position (Fig. 1). The 22,26-dihydroxycholesterol is then hydroxylated at C16 and oxidized at C22, followed by closure of the *E*-ring by *GAME11* and *GAME6* to form the furostanol-type aglycone. This order of reactions is supported by the accumulation of cholestanol-type saponins, lacking hydroxylation at C16 and the hemi-acetal *E*-ring when silencing *GAME11* (fig. S6, F to I). The furostanol-intermediate is oxidized by *GAME4* to its 26-aldehyde, which is the substrate for transamination catalyzed by *GAME12*. Nucleophilic attack of the amino nitrogen at C22 leads to the formation of tomatidenol, which is dehydrogenated to tomatidine. Tomatidine is subsequently converted by *GAME1* to T-Gal (9). T-Gal in its turn is glucosylated by *GAME17* into γ -tomatine, which is further glucosylated by *GAME18* to β 1-tomatine that is finally converted to α -tomatine by *GAME2* (Fig. 1).

Some specialized plant metabolites, particularly terpenoids, are the result of activities from clusters of genes (15, 16). The existence of metabolic gene clusters raises questions about the advantages of such genomic organization (17). Reducing the distance between loci, resulting in coinheritance of advantageous combinations of alleles, may be one benefit of clustering (17). Clustering glycosyltransferases and core pathway genes, as observed here for SGAs, could maintain allelic combinations that support the metabolic outcome needed by the plant and reduce formation of phytotoxic aglycone compounds (9, 18). We found that the regions of coexpressed genes in both chromosomes (i.e., 7 and 12) were flanked by similarly annotated genes and positioned identically along the genome, although poorly coexpressed with *GAME1/SGT1* and *GAME4* and likely not related to SGA metabolism (fig. S11 and table S13). This suggests a duplication event that facilitated the positioning alongside each other on chromosome 12 of *GAME4* and *GAME12*, both STSs and SGAs branch-point genes. Subsequent evolution of enzyme function of this gene pair likely allowed plants in the *Solanaceae* family to start producing the nitrogen-containing steroidal alkaloids.

We have shown that SGA levels can be severely reduced in potato tubers by modifying expression of an enzyme in the biosynthetic pathway. The lack

of SGAs in such plants might make them sensitive to biotic stress, and the increased production of STSs (as occurred in *GAME4*-silenced plants)—which are nontoxic to warm-blooded species, including humans (19)—might provide a compensatory defense mechanism (20). The findings open the way for developing new strategies, through genetic engineering or more classical breeding programs, to reduce quantities of the antinutritional SGAs in key crops of the *Solanaceae*, including potato, tomato, and eggplant. At the same time, it provides a platform for studying the SGA and STS biosynthetic pathways, transport, and regulatory systems that control the production of thousands of these chemicals in specific plant lineages.

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Acknowledgments: We thank A. Tishbee and R. Kramer for operating the UPLC-qTOF-MS instrument and the European Research Council (SAMIT-FP7 program) for supporting the work in the A.A. laboratory. A.A. is the incumbent of the Peter J. Cohn Professorial Chair. J.B. was supported by the European Union 7th Frame Anthocyanin and Polyphenol Bioactives for Health Enhancement through Nutritional Advancement (ATHENA) Project (FP7-KBBE-2009-3-245121-ATHENA). U.H. was partially supported by fellowship AZ: I/82 754, Volkswagen Foundation, Hannover, Germany. We thank the Council of Scientific and Industrial Research (India) for support to A.P.G. (Raman Research Fellowship), A.J.B., Y.C., and P.S. (Research Fellowship) and the University Grants Commission (India) for supporting B.S. We also thank D. R. Nelson for assistance with CYP450 gene classification and R. Last for critically reading the manuscript. A.A. and M.I. are inventors on publication number WO2012095843 A1, submitted by Yeda Research and Development Co. Ltd, which covers the use of the *GAME4* gene for generating low-alkaloid fruit and tubers.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1240230/DC1
Materials and Methods
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Tables S1 to S16
References (21–31)

8 May 2013; accepted 6 June 2013
Published online 20 June 2013;
10.1126/science.1240230

Genome-Wide Comparison of Medieval and Modern *Mycobacterium leprae*

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Leprosy was endemic in Europe until the Middle Ages. Using DNA array capture, we have obtained genome sequences of *Mycobacterium leprae* from skeletons of five medieval leprosy cases from the United Kingdom, Sweden, and Denmark. In one case, the DNA was so well preserved that full de novo assembly of the ancient bacterial genome could be achieved through shotgun sequencing alone. The ancient *M. leprae* sequences were compared with those of 11 modern strains, representing diverse genotypes and geographic origins. The comparisons revealed remarkable genomic conservation during the past 1000 years, a European origin for leprosy in the Americas, and the presence of an *M. leprae* genotype in medieval Europe now commonly associated with the Middle East. The exceptional preservation of *M. leprae* biomarkers, both DNA and mycolic acids, in ancient skeletons has major implications for palaeomicrobiology and human pathogen evolution.

Leprosy, which results from infection with the unculturable pathogen *Mycobacterium leprae*, was common in Europe until the

16th century, when it essentially disappeared. In contrast, disease prevalence has remained high in the developing world. During the past 20 years,

elimination efforts using multidrug therapy have been largely successful, leading to the perception that leprosy is no longer a global health threat despite an annual incidence of over 225,000 cases worldwide (1). To understand the evolution and phylogeography of the leprosy bacillus and to investigate the disappearance of leprosy from Europe, we have used DNA capture techniques and high-throughput sequencing (HTS) to obtain near-complete genome sequences of *M. leprae* from 11th- to 14th-century skeletal remains and from recent biopsies of leprosy patients.

The 3.3 Mb genome of *M. leprae* has undergone massive gene decay (2), and half of the coding potential has been lost, as reflected by the presence of ~1300 pseudogenes. This reductive evolution offers an explanation for the long generation time—~14 days in humans—and our inability to culture the bacillus in vitro (2). The phylogeny of modern *M. leprae* has been investigated by using genome sequencing and a combination of variable number tandem repeat and single-nucleotide polymorphism (SNP) typing, defining four major branches (3–5). However, very little is known about the strains responsible for leprosy in the past. Leprosy is among the few infections that contribute to skeletal changes; hence, attempts to trace its course through history have been made in an archaeological context (6). Molecular biomarkers have permitted detection of *M. leprae* in skeletal remains from many different time periods and geographical locations (7, 8). Studying ancient DNA (aDNA) by using a capture approach has the potential to provide new insight into pathogen evolution, as recently illustrated through full-genome reconstruction of *Yersinia pestis* from skeletal remains (9).

Extracts of bone and teeth from 22 medieval skeletons (Fig. 1, table S1, and supplementary materials notes 1 and 2) with osteological lesions suggestive of leprosy—from cemeteries in Denmark ($n = 8$ extracts) (10), Sweden ($n = 8$ extracts) (7), and the United Kingdom ($n = 6$ extracts) (11)—

were screened for the presence of *M. leprae* DNA by using a bead capture approach of three genomic loci (12). Human mitochondrial DNA (mtDNA) fragments were enriched simultaneously in order to evaluate the characteristic nucleotide misincorporation patterns expected of ancient human (13) and pathogen DNA (14) so as to confirm their authentic ancient origin (supplementary materials note 3). Although DNA damage patterns corresponding to a medieval origin were found in the human mtDNA (15), the captured *M. leprae* DNA revealed much less damage (tables S1 and S2 and figs. S1 to S3), an observation that was entirely unexpected. We therefore relied on traditional authenticity criteria including blank controls, independent replication (16), and other biomarkers, such as mycolic acids (supplementary materials notes 4, 6, and 10; figs. S4 and S5; and tables S4 to S7). Five skeletal samples (3077 from Sweden, Jorgen_625 and Refshale_16 from Denmark, and SK8 and SK14 from the United Kingdom) (table S2) fulfilled our initial criterion for progression to HTS, performed before and after DNA repair (supplementary materials notes 2 and 3). Four samples (3077, Refshale_16, SK8, and SK14) yielded insufficient DNA (table S8) and hence were enriched for *M. leprae* by using DNA array capture (supplementary materials note 5). Un-

expectedly, no enrichment for Jorgen_625 was necessary because an astonishing 40% of the reads mapped to *M. leprae* (table S8). It was thus possible to do a de novo assembly providing over 100-fold genomic coverage (table S9) and 169 contigs, separated by gaps corresponding to repetitive regions (Fig. 2A), that aligned perfectly with the modern *M. leprae* reference genome (supplementary materials note 6) (2). De novo assembly avoids ascertainment biases in gene order that may result from mapping assemblies, although it is often unsuccessful in aDNA investigations owing to inadequate preservation. Few, if any, large insertions or deletions (InDels) have occurred during the ~1000 years that separate the ancient and modern *M. leprae* strains.

To assess the high representation of *M. leprae* sequences in Jorgen_625, the ratios were determined of *M. leprae* versus human sequences in libraries of 230 to 400 base pairs (bp) and 309 to 622 bp fragments (supplementary materials note 5) and found to be 3.3 and 9, respectively (supplementary materials note 6 and fig. S6), thus indicating less fragmentation of the *M. leprae* DNA. The fundamental structural difference between mycobacterial and eukaryotic cells likely accounts for better DNA preservation. Mycobacteria are surrounded by a robust, hydrophobic layer of

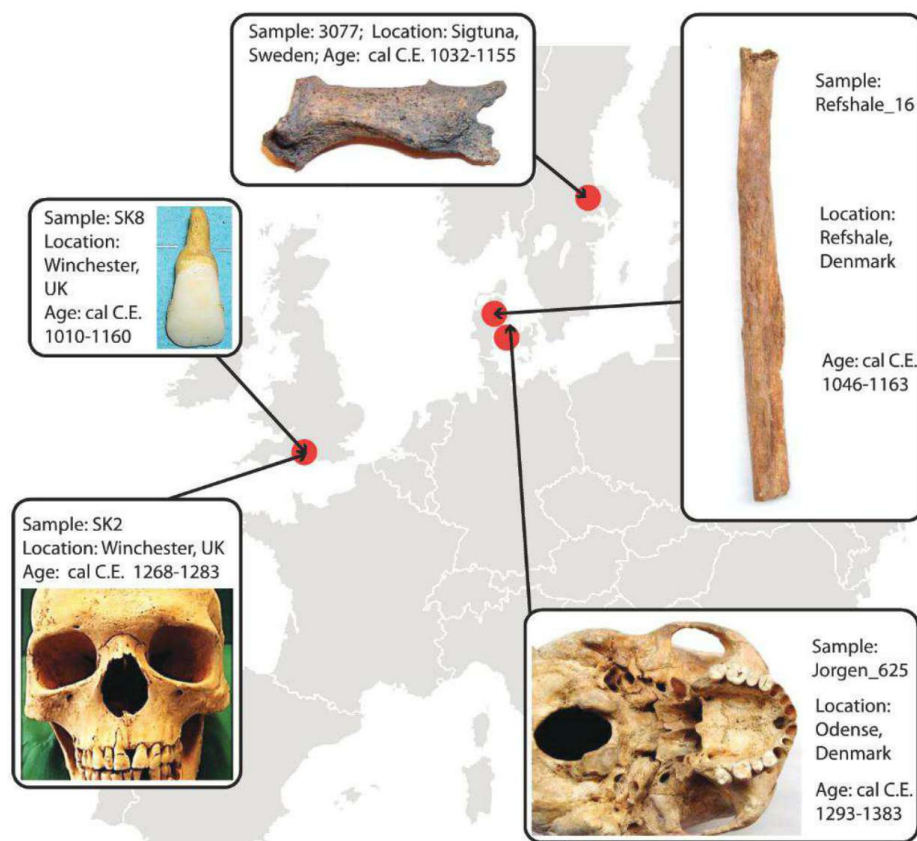


Fig. 1. Sources and origins of the five medieval *M. leprae* strains from Denmark, Sweden, and the United Kingdom for which whole-genome sequences were determined in this study. Pictures of the bones or teeth and their radiocarbon dates are shown.

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mycolic acids that constitute more than 40% of the cell biomass (17). Exceptionally high amounts of mycolic acids that typify *M. leprae* were found in the tooth pulp from Jorgen_625 and, to a lesser extent, in those from SK2, SK8, SK14 (supplementary materials note 10), and Refshale_16 (fig. S5) (11). This finding implies that the lipid-rich cell wall protects mycobacterial DNA (18, 19) from hydrolytic damage (20), hence the longer frag-

ment lengths and reduced nucleotide misincorporation patterns detected. We extended the array-based enrichment and HTS to ancient leprosy specimens (supplementary materials note 5) from Winchester in the United Kingdom (SK2 and SK14) and, as a negative control, a matched skeleton (SK12) from the same cemetery with no osteological evidence of disease. All samples from skeletons

with leprosy-associated lesions (SK2, SK8, and SK14) yielded high-quality *M. leprae* reads from distinct strains. In contrast, <1% of sequences from SK12 mapped to the *M. leprae* genome, mostly to conserved regions present in all mycobacteria (table S10). This analysis further confirmed the authenticity of our samples and eliminated the possibility of cross-contamination among skeletal remains.

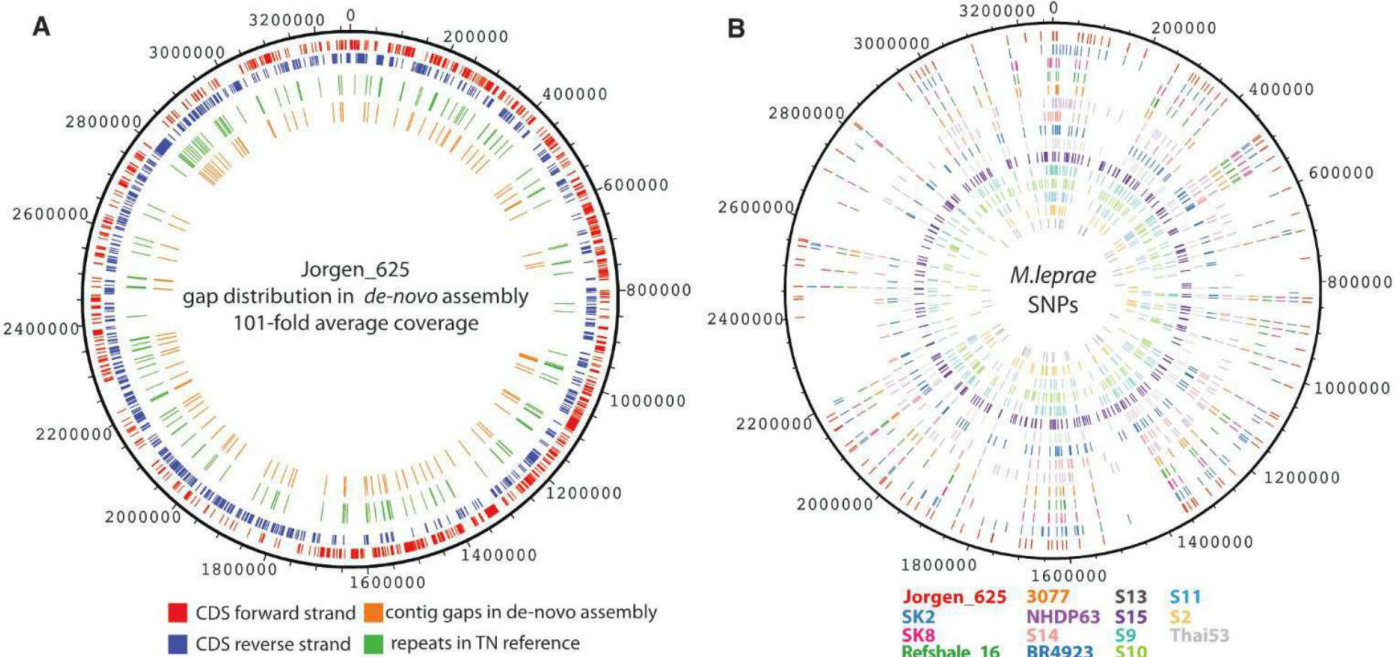


Fig. 2. De novo assembly of the ancient strain of *M. leprae* from skeleton Jorgen_625 and distribution of SNPs across all *M. leprae* genomes sequenced in this study. (A) All the gaps between contigs are in repetitive regions that represent ~2% of the genome of the reference strain TN. There are no structural variations and no changes in synteny, and all the coding sequences in the forward (red) and reverse (blue) strands were as in

the reference genome (metagenomic analysis is available in fig. S9). (B) The 755 SNPs observed among the 16 genomes of *M. leprae* are represented on the 3.27-Mb circular chromosome of the TN genome. The five ancient strains sequenced in this study are shown in the outermost circles followed by 10 modern strains, colored as indicated in the key. Figures were produced with DNAPlotter (29).

Table 1. Ancient and modern strains of *M. leprae* whose genomes were sequenced and compared in this study, together with the reference genomes. BP, before present (in years); Ref, Reference genomes (5).

Sample	Serial number	Name	Radiocarbon date/year of isolation	Percent of genome reconstructed	Average fold coverage	Branch	SNP subtype	Geographic origin
Ancient	3077	3077	938 ± 19 BP	83.77%	10.13	2	2F	Sweden
	JK325	Jorgen_625	644 ± 23 BP	98.26%	101.2	3	3I	Denmark
	JK329	Refshale_16	909 ± 24 BP	97.48%	105.1	2	2F	Denmark
	SK2	SK2	729 ± 23 BP	92.88%	14.87	3	3I	UK
	SK8	SK8	1023 ± 80 BP	96.41%	20.01	2	2F	UK
Modern	S2	95034	1995	84.38%	10.25	1	1B	Antilles
	S9	96008	1996	94.51%	14.45	0	3K	New Caledonia
	S10	Ch-04	2006	96.09%	17.03	0	3K	China
	S11*	Inde 2	1990	97.59%	161.7	1	1D	India
	S13	MI-3-28	2012	93.2%	15.12	4	4N	Mali
	S14	MI-2-7	2012	90.53%	22.4	4	4O	Mali
	S15	92041	1992	91.73%	11.86	4 or 0	3L	New Caledonia
	TN	TN	1990			1	1A	India
Ref*	Thai53	Thai53	1982			1	1A	Thailand
	NHDP63	NHDP63	1996			3	3I	United States
	Br4923	Br4923	1996			4	4P	Brazil

*Passed in armadillos.

A total of five medieval strains—namely 3077, Jorgen_625, Refshale_16, SK2, and SK8—satisfied our criteria for genome-wide comparison (>80% genome coverage, at least fivefold depth), enabling us to compare the genotypes of *M. leprae* strains from 11th- to 14th-century Europe with those of modern strains from different leprosy-endemic regions. For the comparison, the four available reference genome sequences (TN from India, Thai53 from Thailand, NHDP63 from the United States, and Br4923 from Brazil) (5) were supplemented with those of seven modern strains, each belonging to a specific SNP type or of a different geographic origin (Table 1 and table S11), obtained by using multiplexed array capture of DNA prepared directly from skin biopsies of leprosy patients or, in one case, following passage in an armadillo. Here, genome coverage ranged from 83 to 98% at 10- to 160-fold depth (Table 1 and table S9).

Whole-genome reconstructions disclosed a remarkable level of conservation (supplementary materials notes 6 and 9). In total, only 755 SNP and 57 InDels (<7 bp) were found among the 16 *M. leprae* genomes (Fig. 2B and tables S12 to S14). The distribution of the specific SNPs across all 16 genomes revealed 122, 50, and 43 distinct SNPs in modern strains S15, S10, and S9, respectively, accounting for 40% of all the genetic diversity in *M. leprae* known to date. Strains S9 and S15 harbor the Thr⁵³Ile mutation in their *folP1* gene that confers dapsone-resistance (21). No new pseudogenes were found in ancient *M. leprae*, but five were discovered in the modern genomes. These all affect genes for conserved proteins (ML1270, ML1340, ML1761, ML0141, and ML0659) of unknown function.

By far the most polymorphic gene, with a total of 11 SNPs (10 of which are nonsynonymous), is ML0411, which encodes an immunodominant serine-rich cell surface antigen (2), and this may reflect pressure from the host immune system.

The phylogenetic relatedness of all 16 genomes was assessed with outgroup analysis by using maximum parsimony, maximum likelihood, neighbor-joining, and Bayesian phylogeny inference (Fig. 3 and fig. S7). Most strains clustered into four major branches—which is consistent with the SNP typing scheme (5)—except for S9 and S10, which form the deepest lineages, and a new branch, named branch 0 (Table 1). Strain S15 also displayed deep divergence and could not be unambiguously placed among the five branches (Fig. 3). These three strains (S9, S10, and S15) were found to branch off closest to the common ancestor of *M. leprae*, *M. tuberculosis*, *M. ulcerans*, and *M. avium* (supplementary materials note 7 and figs. S7 and S8).

Two new phylogeographic conclusions can be drawn from our reconstructed *M. leprae* phylogeny. First, three ancient strains (3077, Refshale_16, and SK8) belonging to branch 2 form a tight cluster with very short branch lengths (Fig. 3A). Modern branch 2 strains have previously been reported only in Iran and Turkey (5), pointing to a possible link between Middle-Eastern and medieval European strains. Second, branch 3 strains have not been found in the Middle East (5), whereas this genotype has been detected in European skeletons (5, 7, 8), including SK2. The striking closeness of Jorgen_625 and SK2 (Fig. 3A) with the NHDP63 strain, and 52 other branch 3 strains from the United States (22), is consistent with the European origin of leprosy in the Americas (4).

Branch shortening (supplementary materials note 8) is commonly observed when ancient sequences are included in tree reconstructions owing to their lower number of derived positions. The five ancient *M. leprae* strains do indeed have shorter branch lengths than those of modern strains, which have accumulated more substitutions. The average distance of the ancient strains to the most recent common ancestor (MRCA) was 19.8 nucleotides, whereas it was 27.5 nucleotides for the modern strains, a statistically significant difference (Mann-Whitney *U* test: $U = 7.50$, $P < 0.05$).

Divergence times for the *M. leprae* strains were estimated by using the Bayesian inference software BEAST (23), with models of both a strict and a relaxed clock in order to compensate for possible rate variation among lineages observed in other bacterial pathogens (24). For both models, radiocarbon dates for ancient skeletal samples and isolation dates for the modern bacteria were used as tip calibration (supplementary materials note 8). Using the relaxed clock and 16 *M. leprae* genomes, we estimated a rate of 8.6×10^{-9} substitutions per site per year [1.32×10^{-8} to 3.61×10^{-9} 95% highest posterior density (HPD)]. Exclusion of S15 permitted the use of a strict clock, yielding an estimated rate of 6.13×10^{-9} substitutions per site per year (8.56×10^{-9} to 3.38×10^{-9} 95% HPD). The calculated mutation rate of *M. leprae* by use of direct fossil calibration is thus close to that of the related human pathogen *M. tuberculosis* by use of tip calibrations from modern strains [5.4×10^{-9} substitutions per site per year (24)]. It remains to be seen whether long-time fossil calibration as applied here to *M. leprae* will produce similar results for *M. tuberculosis*. The resulting divergence

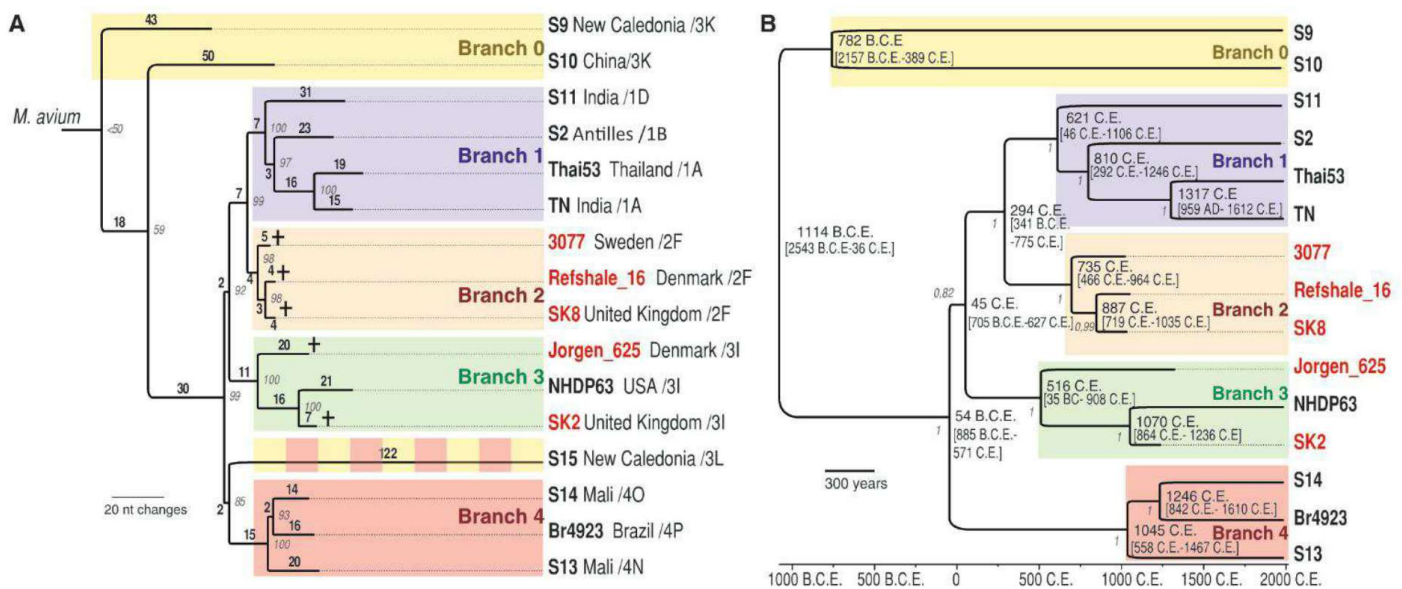


Fig. 3. Phylogeny of medieval and modern *M. leprae*. (A) Phylogenetic relationship of *M. leprae* genomes using a maximum parsimony tree, including *M. avium* as an outgroup. Geographic origin and SNP type are given at each branch tip. Bootstrap node support is shown in gray, and nucleotide substitutions on each branch is in bold. (B) Bayesian phylogenetic tree

calculated with BEAST 1.7.1 (23), including all ancient strains with radiocarbon dates, inferred from a total of 516 genome-wide variable positions. Divergence time intervals are shown on each node in years B.C.E. and C.E. Posterior probabilities for each node are shown in gray. Labeling colors vary from red to blue based on the tip age for each branch.

times for the MRCA for all the *M. leprae* strains are 2871 years ago (1350 to 5078 years ago) and 3126 years ago (1975 to 4562 years ago) for the relaxed and strict clock models, respectively (Fig. 3B). This is consistent with the oldest known and accepted osteological evidence for leprosy from 2000 BCE. India (25).

The sudden decline of leprosy in 16th-century Europe was almost certainly not due to the medieval European strain of *M. leprae* losing virulence, as evidenced by the close similarity between the modern NHDP63 strain and ancient Jorgen_625 and SK2 strains. Extraneous factors—for example, other infectious diseases such as plague or tuberculosis, changes in host immunity (26, 27), or improved social conditions—may have accounted for its decline. Our finding of well-preserved mycolic acids in a 14th-century tooth together with sufficient DNA to generate a whole de novo genome sequence may enable investigation of ancient leprosy bacilli and other pathogens well beyond the inferred maximum age for mammalian DNA of around 1 million years (28). There is thus a real prospect that the prehistoric origins of *M. leprae* can be retraced.

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Acknowledgments: We are grateful to the following people for providing samples, support, and advice: C. Bauer, H. Burbano, J. Hinds, S. Junker, M. Kodio, A. Fomba, C. Lanz, P. McLaren, J. Rougemont, and S. Schreiber. All raw read files have been deposited in the trace archive of the National Center for Biotechnology Information Sequence Read Archive under accession no. SRP022139. This work was supported by the European Research Council (ERC-APGREID), the Carl Zeiss Foundation, the Fondation Raoul Follereau, the Swiss National Science Foundation (Brazilian Swiss Joint Research Program), the Deutsche Forschungsgemeinschaft Priority Program 1335 Scalable Visual Analytics, the Central Innovation Program (grant KF2701103BZ1), the Graduate School Human Development in Landscapes, the Excellence Cluster Inflammation at Interfaces, the Medical Faculty of the Christian-Albrechts-University Kiel, a British Academy Small Research Grant, the Leverhulme Trust (Grant F/00094/BL), and the Social Sciences and Humanities Research Council of Canada (postdoctoral fellowship grant 756-2011-0501). Human remains were obtained under license from the UK Ministry of Justice.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1238286/DC1
Materials and Methods

Figs. S1 to S9

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References (30–73)

25 March 2013; accepted 28 May 2013

Published online 13 June 2013;

10.1126/science.1238286

Infectivity, Transmission, and Pathology of Human-Isolated H7N9 Influenza Virus in Ferrets and Pigs

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The emergence of the H7N9 influenza virus in humans in Eastern China has raised concerns that a new influenza pandemic could occur. Here, we used a ferret model to evaluate the infectivity and transmissibility of A/Shanghai/2/2013 (SH2), a human H7N9 virus isolate. This virus replicated in the upper and lower respiratory tracts of the ferrets and was shed at high titers for 6 to 7 days, with ferrets showing relatively mild clinical signs. SH2 was efficiently transmitted between ferrets via direct contact, but less efficiently by airborne exposure. Pigs were productively infected by SH2 and shed virus for 6 days but were unable to transmit the virus to naïve pigs or ferrets. Under appropriate conditions, human-to-human transmission of the H7N9 virus may be possible.

On 31 March 2013, the Chinese National Health and Family Planning Commission announced the occurrence of three human infections with H7N9 subtype influenza viruses (1). Analyses of the sequences of the human H7N9 isolates indicate that the virus was derived by reassortment events between H7 and N9 subtype viruses, possibly from aquatic birds, and enzootic H9N2 viruses from chickens (1, 2). As of 1 May 2013, more than 125 human cases

have been confirmed, with the majority of the patients hospitalized and many suffering acute respiratory distress syndrome (3–5). More than 75% of human cases had a history of contact with, or exposure to, poultry before disease onset (4), suggesting a zoonotic origin of the infections. Identification of some family clusters raised concerns of human-to-human transmission by the H7N9 virus (4). Sequence analyses showed that the H7N9 viruses might have undergone mutations that are

favorable for efficient replication in mammalian hosts (2, 6–8).

To characterize this H7N9 virus, we assessed its infectivity, transmissibility, and pathogenicity in ferrets, the primary mammalian model for human influenza. Influenza-free ferrets ($n = 6$) were inoculated intranasally with 10^6 times the median tissue culture infectious dose (TCID₅₀) of A/Shanghai/02/2013 (SH2), a human isolate from a fatal index case (see supplementary materials and methods) (1). These ferrets displayed a brief fever at 1 to 2 days postinoculation (dpi) and robust sneezing and nasal discharge throughout the experiment (Table 1 and fig. S1, A, C, and D). Coughing and mild lethargy occurred

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periodically during the course of the disease, but weight change was essentially negligible (fig. S1, B, E, and F). Ferrets ($n = 6$) inoculated with the pandemic A (H1N1) 2009 virus, A/California/07/2009 (CA07), showed similar signs as the SH2-inoculated animals, except for more prominent nasal discharge and slightly greater weight loss, but without statistically significant differences (fig. S1). All animals in both virus groups displayed near-normal activity levels by 14 dpi but had some residual sneezing and nasal discharge. SH2-infected animals were normal for body temperature, body weight, sneezing, coughing, and activity by 16 dpi (table S1).

The virus load in the nasal washes of each inoculated ferret was determined daily by TCID₅₀ assays in Madin-Darby Canine Kidney (MDCK) cells. Virus shedding was detected at 1 dpi and was sustained at high titers (3.1 to 5.4 log TCID₅₀/ml)

for 7 days (Fig. 1A and Table 1). Thus, virus shedding occurred before the development of most clinical signs. For the CA07-infected ferrets, virus shedding began at 1 dpi and continued at high titers (3.1 to 5.9 log TCID₅₀/ml) for 6 days (Fig. 1A). Overall, SH2 and CA07 infection showed comparable clinical profiles and virus shedding kinetics in ferrets with no statistically significant differences.

Efficient transmission of influenza viruses in ferrets is considered as a predictor of human-to-human transmissibility (9). Six ferrets inoculated with 10⁶ TCID₅₀ of virus were placed, two each, in three transmission cages. At 1 dpi, a naïve ferret was introduced into each cage with the inoculated ferrets to measure direct-contact transmission (fig. S2A). An additional naïve ferret was placed in an adjacent cage, separated by a distance of 10 cm for airborne exposure (fig. S2A), with

airflow toward this cage at a rate of <0.2 m/s. An identical experiment was conducted with the CA07 virus.

All three direct-contact ferrets of the SH2 inoculated group shed virus within 3 days post-exposure (dpe) and showed sneezing, nasal discharge, coughing, and inactivity by 6 dpe, but only one developed fever for 1 day at 4 dpe (Fig. 1B and Table 1). One of the airborne-exposed ferrets began shedding virus at 3 dpe and continued shedding virus at high titers for 6 days (Fig. 1C and Table 1). The two remaining airborne-exposed ferrets did not shed detectable virus and had few clinical signs (Fig. 1C and Table 1). All inoculated and direct-contact ferrets seroconverted by 14 dpi or 14 dpe. The airborne-exposed ferret that shed virus seroconverted with an hemagglutination inhibition (HAI) titer of 1:320, whereas one airborne-exposed ferret that did not shed virus had an HAI titer of 1:40 at 14 dpe (Table 1). Thus, ferrets infected with SH2 can transmit the virus via direct contact and airborne exposure, albeit the latter less efficiently. All naïve ferrets placed in CA07 direct-contact and airborne-exposure cages began shedding virus between 1 and 4 dpe (Fig. 1, D and E, and Table 1), consistent with earlier studies (10, 11).

Eighteen ferrets inoculated with SH2 as above were killed at 1, 3, 5, 7, 10, and 14 dpi to examine the gross pathology and infected tissue types to study disease progression. Respiratory and other major organs were collected for histopathologic examination and immunostaining for the presence of viral nucleoprotein (NP). Virus load was also determined by detection of a viral matrix gene by real-time polymerase chain reaction (RT-PCR) and TCID₅₀ assays.

Table 1. Transmissibility of SH2 and CA07 viruses in ferrets. nd, not detectable; na, not applicable.

Ferret groups	Virus*	Signs	Shedding of virus			Seroconversion‡
			No. of animals	Onset†	Duration (days)	No. of animals (titers)
Inoculated	SH2	6/6	6/6	1,1,1,1,1,1	7,7,7,7,7,7	6/6 (320–640)
	CA07	6/6	6/6	1,1,1,1,1,1	5,6,6,6,6,6	6/6 (>1280)
Direct contacts	SH2	3/3	3/3	2,3,3	5,6,7	3/3 (320–640)
	CA07	3/3	3/3	1,1,2	6,6,6	3/3 (>1280)
Airborne-exposed	SH2	3/3	1/3	nd,nd,3	na,na,6	2/3 (320, 40, <10)
	CA07	3/3	3/3	2,3,4	6,6,7	3/3 (>1280)

*SH2, A/Shanghai/2/2013(H7N9); CA07, A/California/07/2009 (H1N1). †dpi, dpe. ‡HAI titer of postexposure sera at 14 dpi or 14 dpe for each animal, starting dilution at 1:10.

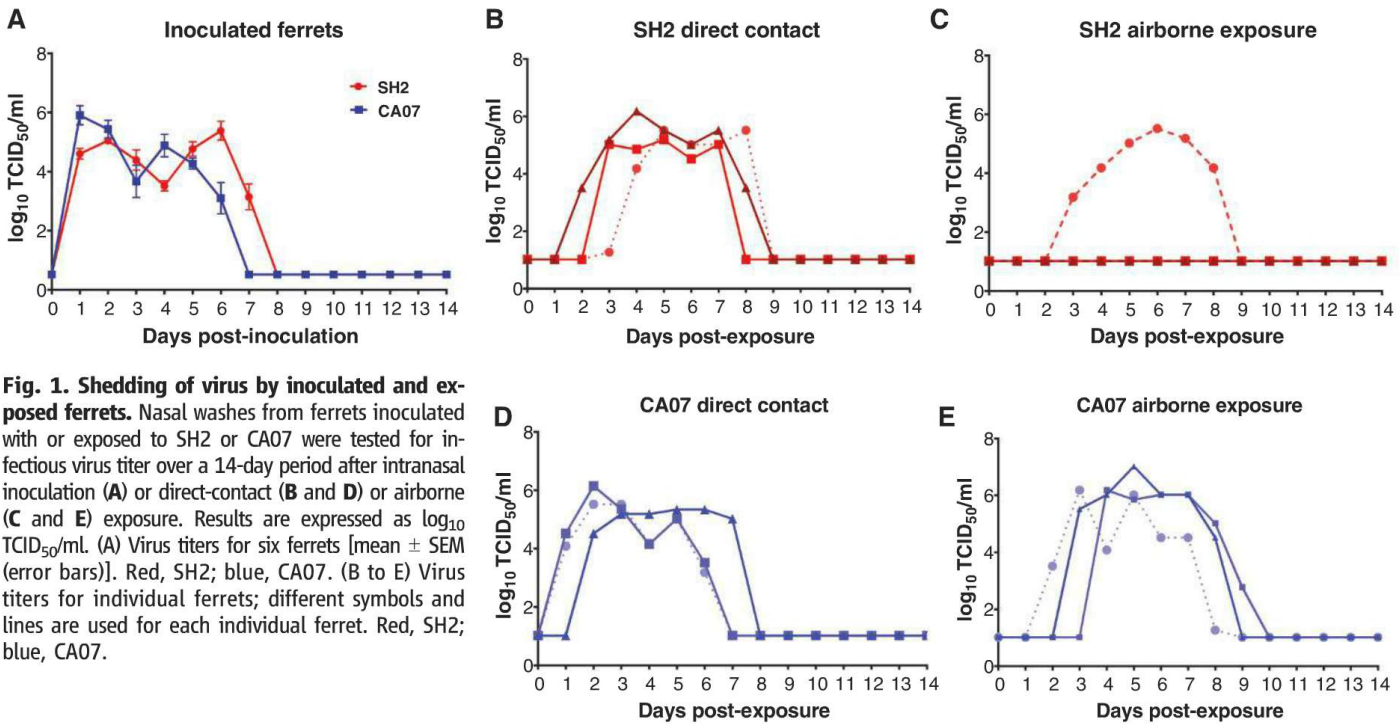


Fig. 1. Shedding of virus by inoculated and exposed ferrets. Nasal washes from ferrets inoculated with or exposed to SH2 or CA07 were tested for infectious virus titer over a 14-day period after intranasal inoculation (A) or direct-contact (B and D) or airborne (C and E) exposure. Results are expressed as log₁₀ TCID₅₀/ml. (A) Virus titers for six ferrets [mean ± SEM (error bars)]. Red, SH2; blue, CA07. (B to E) Virus titers for individual ferrets; different symbols and lines are used for each individual ferret. Red, SH2; blue, CA07.

NP-positive cells were detected in the respiratory epithelial cells of the nasal turbinate and trachea at 3 dpi (Fig. 2, A and B). Bronchiolar epithelial cells were also positive for NP at 3 dpi (Fig. 2C). Gross pathological examination of the lungs revealed multifocal lesions on days 3, 5, and 7. At 3 dpi, histopathological examinations showed focal bronchopneumonia with acute neutrophil-predominant inflammatory infiltrates in the bronchioles and alveoli (Fig. 2, D and G). By 5 and 7 dpi, some acute but predominantly chronic lymphoplasmacytic inflammatory infil-

tration was seen in the bronchioles and alveoli (Fig. 2, E, F, and H).

Viral RNA was detected in ferret nasal turbinate, trachea, lungs, hilar lymph nodes, and brain (Fig. 3). The presence of infectious virus was confirmed by TCID₅₀ assays (fig. S3) and NP staining in the brain, although this was predominantly cytoplasmic, and in hilar lymph nodes (fig. S4). Thus, inoculation of SH2 can result in infection of the upper and lower respiratory tracts, lymph nodes, and, potentially, the brain. A different site of inoculation, such as the trachea (12),

may result in a different pathology. Clinically, the cellular tropism of H7N9 viruses may determine its spectrum of clinical disease, and it may be advisable to examine human cases for signs of central nervous system affects.

The domestic pig is a major mammalian host of influenza A viruses and may have played a key role in facilitating the emergence of human pandemic influenza viruses (13). To evaluate the infectivity and transmissibility of SH2 in pigs, four animals were inoculated with 10⁶ TCID₅₀ of SH2. Virus shedding was detected

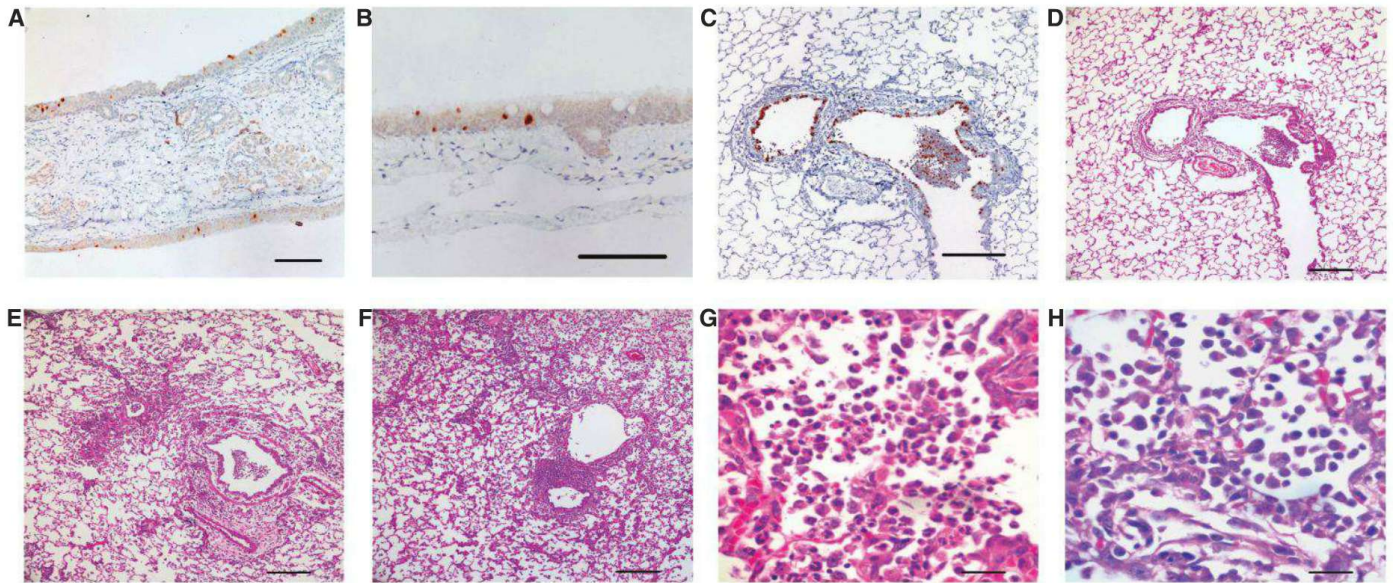


Fig. 2. Histopathology and immunohistochemical analyses of ferret respiratory tissues after SH2 infection. Influenza NP antigen staining is visible (brown) in the nasal turbinate (A), trachea (B), and lung (C) at 3 dpi. Pulmonary tissues were harvested from ferrets infected with SH2 and hematoxylin-and-eosin stained at 3 (D), 5 (E), and 7 dpi (F). Pathological

changes characteristic of bronchopneumonia with mixed inflammatory infiltrates of bronchioles and alveoli were observed. At 3 dpi, neutrophils were the predominant infiltrating cell type (G). By 5 dpi, chronic lymphoplasmacytic infiltrates are more prominent (H). Scale bars indicate 100 μ m (A and B), 200 μ m (C to F), or 20 μ m (G and H).

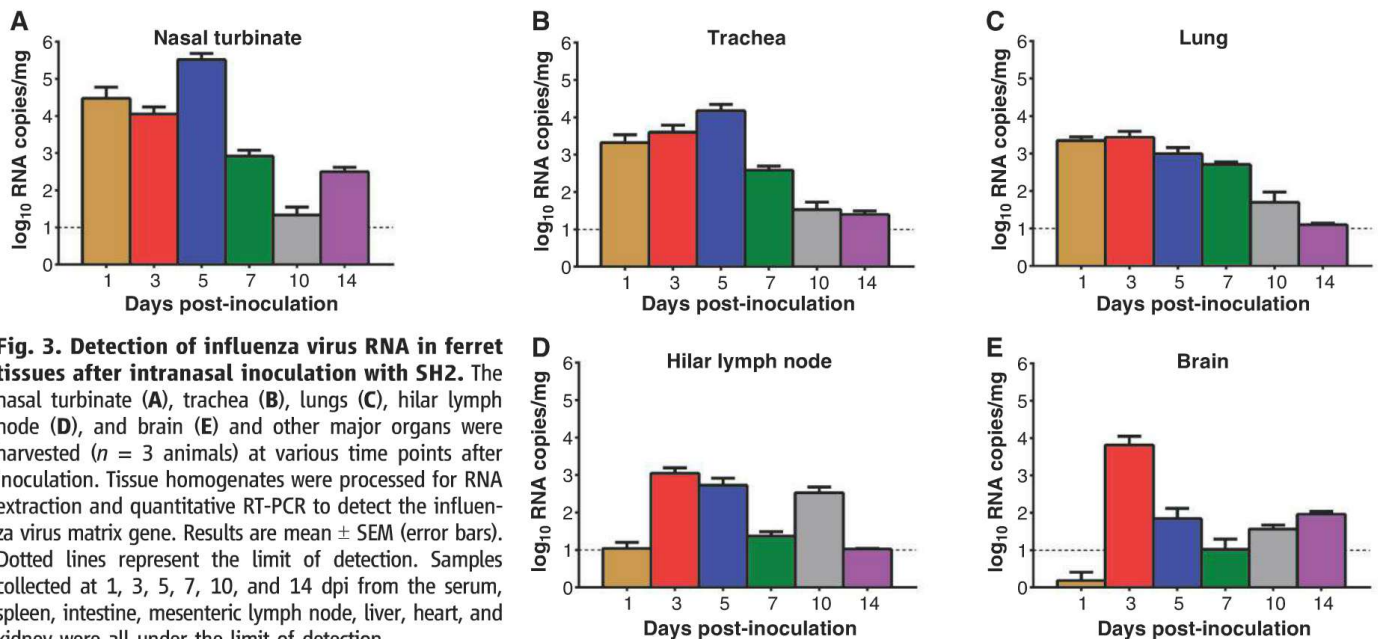


Fig. 3. Detection of influenza virus RNA in ferret tissues after intranasal inoculation with SH2. The nasal turbinate (A), trachea (B), lungs (C), hilar lymph node (D), and brain (E) and other major organs were harvested ($n = 3$ animals) at various time points after inoculation. Tissue homogenates were processed for RNA extraction and quantitative RT-PCR to detect the influenza virus matrix gene. Results are mean $\pm$ SEM (error bars). Dotted lines represent the limit of detection. Samples collected at 1, 3, 5, 7, 10, and 14 dpi from the serum, spleen, intestine, mesenteric lymph node, liver, heart, and kidney were all under the limit of detection.

Table 2. Transmission of SH2 and CA07 in pigs and to ferrets. nd, not detectable; na, not applicable.

Infection group	Virus*	Shedding of virus			Seroconversion‡
		No. of animals	Onset†	Duration (days)	No. of animals (titers)
Inoculated pigs	SH2	4/4	1,2,2,2	5,5,5,6	3/4 (10,40,80,640)
	CA07	4/4	1,1,1,1	6,6,6,7	3/4 (20,40,80,80)
Direct-contact pigs	SH2	0/4	nd,nd,nd,nd	na,na,na,na	1/4 (<10,<10,<10,320)
	CA07	4/4	3,3,3,4	5,6,6,7	4/4 (40,40,80,80)
Airborne-exposed pigs	SH2	0/2	nd,nd	na,na	0/2 (<10,<10)
	CA07	2/2	3,4	5,6	2/2 (40,160)
Airborne-exposed ferrets	SH2	0/2	nd,nd	na,na	2/2 (40,320)
	CA07	2/2	6,7	7,7	2/2 (>1280, >1280)

*SH2, A/Shanghai/2/2013(H7N9); CA07, A/California/07/2009 (H1N1). †dpi, dpe. ‡HA1 titer of postexposure sera at 14 dpi or 14 dpe for each animal, starting dilution at 1:10.

as early as 1 dpi and lasted for 5 to 6 days, with peak virus titers ranging from 3.49 to 5.16 log TCID₅₀/ml (Table 2 and fig. S5A). Sneezing, nasal discharge, and diminished activity were observed from 1 to 1.5 days after virus shedding.

To determine transmissibility, at 1 dpi, two naïve pigs were housed with each of the two groups of inoculated animals, and a further naïve pig and ferret were placed in separate cages to assess airborne transmission (fig. S2B). None of the direct-contact pigs or airborne-exposed pigs and ferrets shed virus via the nasal or rectal routes (Table 2 and fig. S5, C and D). One of the four direct-contact pigs and both airborne-exposed ferrets seroconverted by 14 dpe. Neither airborne-exposed pig seroconverted (Table 2). Three additional inoculated pigs were killed at 4 dpi to detect the presence of virus in major organs. Viral RNA was detected in the nasal turbinate, trachea, lungs, and lymph nodes of these pigs and NP in nasal turbinates (fig. S6). Additionally, viral RNA was detected in the kidney, heart, liver, spleen, and intestine, albeit at levels closer to the detection limit. Thus, SH2 could productively infect domestic pigs after intranasal inoculation. An identical transmission experiment using the CA07 virus showed that all direct-contact pigs and airborne-exposed pigs and ferrets could shed virus (Table 2 and fig. S5, B to D).

Thus, ferrets can be infected by SH2 and can shed virus that may transmit to direct-contact and airborne-exposed animals, resulting in productive infections. Shedding of this virus occurred before the development of the majority of clinical signs. This trait has been observed previously for pandemic and seasonal influenza (10, 11, 14, 15). If this virus acquires the ability to efficiently transmit from human to human, extensive spread of this virus may be inevitable, as quarantine measures will lag behind its spread. Assuming that poultry is the source of the H7N9 virus, continued prevalence of this virus could lead to it becoming enzootic in poultry, as has occurred with the Asian highly pathogenic H5N1 and H9N2 virus lineages (16, 17). If so, the op-

portunities for the H7N9 virus to evolve to acquire human-to-human transmissibility, or to be introduced to pigs, would greatly increase. To prevent this from happening, it may be advisable to reconsider the management of live poultry markets, especially in the urban areas.

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Acknowledgments: We gratefully acknowledge our colleagues from the Joint Influenza Research Center (SUMC/HKU) and the State Key Laboratory of Emerging Infectious Diseases for their excellent technical assistance and D. K. Smith for editorial assistance. This study was supported by the NIH (National Institute of Allergy and Infectious Diseases contract HSN266200700005C), Li Ka Shing Foundation, the Area of Excellence Scheme of the University Grants Committee of the Hong Kong SAR (grant AoE/M-12/06), Shenzhen Peacock Plan High-End Talents Program (KQTD201203), and Emergency Research Project on human infection with avian influenza H7N9 virus from the National Ministry of Science and Technology (no. KJYJ-2013-01-01-01 to Y.S.). The sequences generated by this study were deposited in GenBank under accession nos. KF0211594 to KF0211601; other data can be found in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/science.1239844/DC1
Materials and Methods
Figs. S1 to S6
Table S1
References (18–21)
30 April 2013; accepted 20 May 2013
Published online 23 May 2013;
10.1126/science.1239844

Mg²⁺ Regulates Cytotoxic Functions of NK and CD8 T Cells in Chronic EBV Infection Through NKG2D

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The magnesium transporter 1 (MAGT1) is a critical regulator of basal intracellular free magnesium (Mg²⁺) concentrations. Individuals with genetic deficiencies in *MAGT1* have high levels of Epstein-Barr virus (EBV) and a predisposition to lymphoma. We show that decreased intracellular free Mg²⁺ causes defective expression of the natural killer activating receptor NKG2D in natural killer (NK) and CD8⁺ T cells and impairs cytolytic responses against EBV. Notably, magnesium supplementation in *MAGT1*-deficient patients restores intracellular free Mg²⁺ and NKG2D while concurrently reducing EBV-infected cells in vivo, demonstrating a link between NKG2D cytolytic activity and EBV antiviral immunity in humans. Moreover, these findings reveal a specific molecular function of free basal intracellular Mg²⁺ in eukaryotic cells.

Divalent metal cations, including Mg²⁺, play important roles in cellular processes. In eukaryotic cells, 95% of intracellular Mg²⁺ ([Mg²⁺]_i) is bound (1). The remaining un-

bound, free [Mg²⁺]_i (~0.5 to 1 mM) is tightly regulated, yet its specific molecular functions are unknown (1–3). Magnesium transporter 1 (*MAGT1*) selectively conducts Mg²⁺ across the plasma mem-

brane (4, 5). We previously characterized a primary immunodeficiency, named X-linked immunodeficiency with Mg^{2+} defect, Epstein-Barr virus (EBV) infection, and neoplasia (XMEN) disease, caused by null mutations in the *MAGT1* gene. We found that *MAGT1* was required for a T cell

receptor (TCR)-stimulated Mg^{2+} influx that transiently raises free $[Mg^{2+}]_i$ to temporally coordinate T cell activation (2, 3).

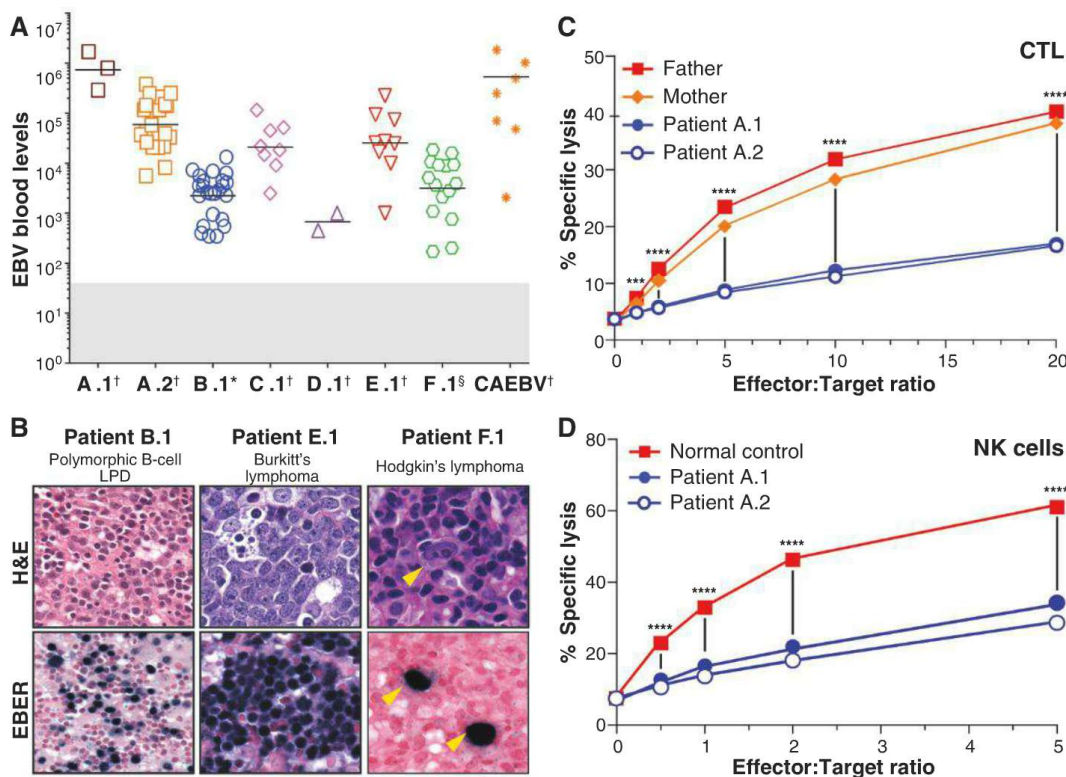
XMEN disease is characterized by uncontrolled chronic EBV infection (2, 3). EBV is a B cell-tropic gammaherpesvirus that infects and establishes latency in 90% of humans worldwide (6). Uncontrolled EBV infection can lead to malignancy, especially in B cells (7–9). The cytotoxic functions of natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) are essential for control of viral infections and tumor immunosurveillance (10). XMEN patient cells contain normal bound $[Mg^{2+}]_i$ but decreased free $[Mg^{2+}]_i$ (2, 3), and whether the latter contributes to XMEN disease pathogenesis was unknown.

To understand defective EBV control in XMEN disease, we examined seven patients harboring mutations in *MAGT1* (table S1). Similar to the first three patients characterized (A.1, A.2, and B.1) (2, 3), four additional XMEN patients with repeated minor viral infections and elevated EBV DNA in the blood were identified (table S1 and Fig. 1A). Furthermore, starting in childhood, some patients developed various EBV⁺ Hodgkin's, Burkitt's, and non-Hodgkin's B cell lymphomas or related lymphoproliferative disorders (table S1 and Fig. 1B). Nevertheless, XMEN patients had normal adaptive immune responses, generating EBV-specific memory $CD8^+$ T cells (fig. S1). However, unlike normal controls, EBV-specific

CTLs from XMEN patients expanded by serial stimulations with irradiated autologous lymphoblastoid cell lines (LCLs) showed defective cytotoxicity against autologous LCLs (Fig. 1C) despite comparable expansions of EBV-tetramer-positive cells (fig. S2). Moreover, NK cells that were expanded in vitro with interleukin-2 (IL-2) exhibited defective cytotoxicity against K562 target cells (Fig. 1D). The NK cytotoxicity defect indicated that the previously described TCR defect could not fully account for the defective EBV immunity in XMEN patients. Decreased cytotoxicity also did not stem from deficient granule effectors or degranulation (fig. S3).

MAGT1 not only mediates TCR-induced Mg^{2+} flux but also regulates the basal free $[Mg^{2+}]_i$ (2–4, 11, 12). We found that all XMEN patients, but not chronic active EBV (CAEBV) patients lacking *MAGT1* mutations or X-linked lymphoproliferative disease (XLP) patients chronically infected with EBV, had decreased basal free $[Mg^{2+}]_i$ (Fig. 2, A and B). Given that the bound $[Mg^{2+}]_i$ (95 to 99% of total) is not reduced in XMEN patients (3, 5), we hypothesized that Mg^{2+} supplementation might restore the free $[Mg^{2+}]_i$ in XMEN patient cells. We therefore cultured CTLs and NK cells from XMEN patients in Mg^{2+} -supplemented media and observed a dose-dependent increase in free $[Mg^{2+}]_i$ with nearly full restoration after 5 days in 10 mM magnesium sulfate ($MgSO_4$) (Fig. 2, C and D).

Fig. 1. XMEN patients exhibit high EBV levels, lymphoma development, and defective cytotoxicity. (A) Levels of EBV DNA in XMEN patient blood ($n = 7$) were expressed as copies/ml blood (†), copies/ 10^6 cells (*), or copies/ μ g DNA (§) depending on the specific clinical laboratory. Each point represents an independent blood draw measurement at different times. Chronic active EBV (CAEBV) patient values are shown for comparison. Shaded area indicates normal range; EBV DNA is typically undetectable in EBV⁺ subjects, with less than 10% of normal individuals usually showing no more than 20 to 80 copies/ml in the blood (37). (B) Histopathology of the various EBV⁺ lymphoproliferative disorders (LPD) in XMEN patients. The upper panel shows the hematoxylin and eosin (H&E) staining, and the lower panel shows the EBV-encoded small RNA (EBER) staining by in situ hybridization. Yellow arrowheads show Reed-Sternberg cells. Magnification: $\times 400$ for patients B.1 and E.1 (bottom panel) and $\times 1000$ for patient F.1 and E.1 (top panel). (C) Cytotoxicity of EBV-specific CTLs from father (squares), mother (diamonds), and XMEN patients A.1 (filled circles) and A.2 (open circles) on autologous LCLs. (D) Cytotoxicity of IL-2-expanded NK cells from normal control (filled squares) or XMEN patients A.1 (filled



circles) and A.2 (open circles) against K562 cells. Results shown in (C) and (D) are mean $\pm$ SEM and are representative of all XMEN patients tested ($n = 4$) in at least three independent experiments [two-way analysis of variance (ANOVA), *** $P < 0.001$; **** $P < 0.0001$].

circles) and A.2 (open circles) against K562 cells. Results shown in (C) and (D) are mean $\pm$ SEM and are representative of all XMEN patients tested ($n = 4$) in at least three independent experiments [two-way analysis of variance (ANOVA), *** $P < 0.001$; **** $P < 0.0001$].

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Magnesium supplementation did not rescue the TCR activation defect (Fig. 2E) or TCR-induced Mg^{2+} and Ca^{2+} fluxes in XMEN T cells (fig. S4). However, Mg^{2+} supplementation did recover the cytotoxicity defect partially in CTLs and nearly completely in NK cells (Fig. 2, F and G), suggesting that the chronically low free $[Mg^{2+}]_i$ caused impaired cytotoxicity.

NK cells use a combination of inhibitory and activating receptors to specifically recognize target cells altered by transformation or virus infection (13, 14). Certain activating NK receptors are also expressed on CTLs as costimulatory signals for cytotoxic function (15). We surveyed the major NK receptors and found a selective loss of NKG2D expression on XMEN CTLs and NK cells (Fig. 3, A and B). This was confirmed by surface staining, intracellular staining, and immunoblotting (Fig. 3, A to C, and fig. S5A). The NKG2D loss was not secondary to EBV infection, as cells from CAEBV or XLP patients expressed normal NKG2D levels (Fig. 3B). NKG2D is

associated with the adapter DAP10 on NK cells, CTLs, and $\gamma\delta$ T cells and mediates antiviral and antitumor cytotoxic responses (16–20). We also observed decreased DAP10 in XMEN patients, which could be a cause or consequence of the NKG2D defect (20) (Fig. 3C). Their defective expression was posttranscriptional, as their steady-state mRNA levels were not reduced in XMEN samples (fig. S5B). Ubiquitin-conjugated peptides of NKG2D and DAP10 were detected by mass spectrometry, implying accelerated protein turnover.

In humans, NKG2D ligands include the major histocompatibility complex (MHC) class I-like proteins MICA and MICB, as well as the UL16-binding protein family of glycoproteins (ULBP1–6) that are induced by infection, cellular transformation, and cell stress (19, 21, 22). Using a ULBP1-expressing P815 target cell, we observed that NKG2D-mediated cytotoxicity by XMEN patient NK cells was abolished (Fig. 3D). Similarly, although defective cytotoxicity against P815 cells coated with an antibody to CD3 (anti-CD3) could

involve the TCR signaling defect previously documented, further enhancement of the anti-CD3–induced cytotoxicity by ULBP1 was defective (Fig. 3E), suggesting that NKG2D loss contributed to the CTL killing defect in XMEN patients (2, 3).

We next examined Mg^{2+} and Ca^{2+} fluxes after stimulating various NK receptors and found that only CD16 stimulation induced a Mg^{2+} flux (fig. S6A). This flux was MAGT1-dependent and absent in XMEN NK cells but did not affect the Ca^{2+} flux in XMEN samples (fig. S6A). Most NK receptors work in synergy with each other (23), and we consistently observed a defect when triggering flux through combinations that included NKG2D in XMEN patient cells (fig. S6, B and C).

Because NKG2D was constitutively reduced in XMEN cells, we hypothesized that this resulted from decreased free $[Mg^{2+}]_i$. Correspondingly, we found that cultivation of normal CTLs in Mg^{2+} -depleted medium caused a reduction of free $[Mg^{2+}]_i$ and NKG2D expression (fig. S7).

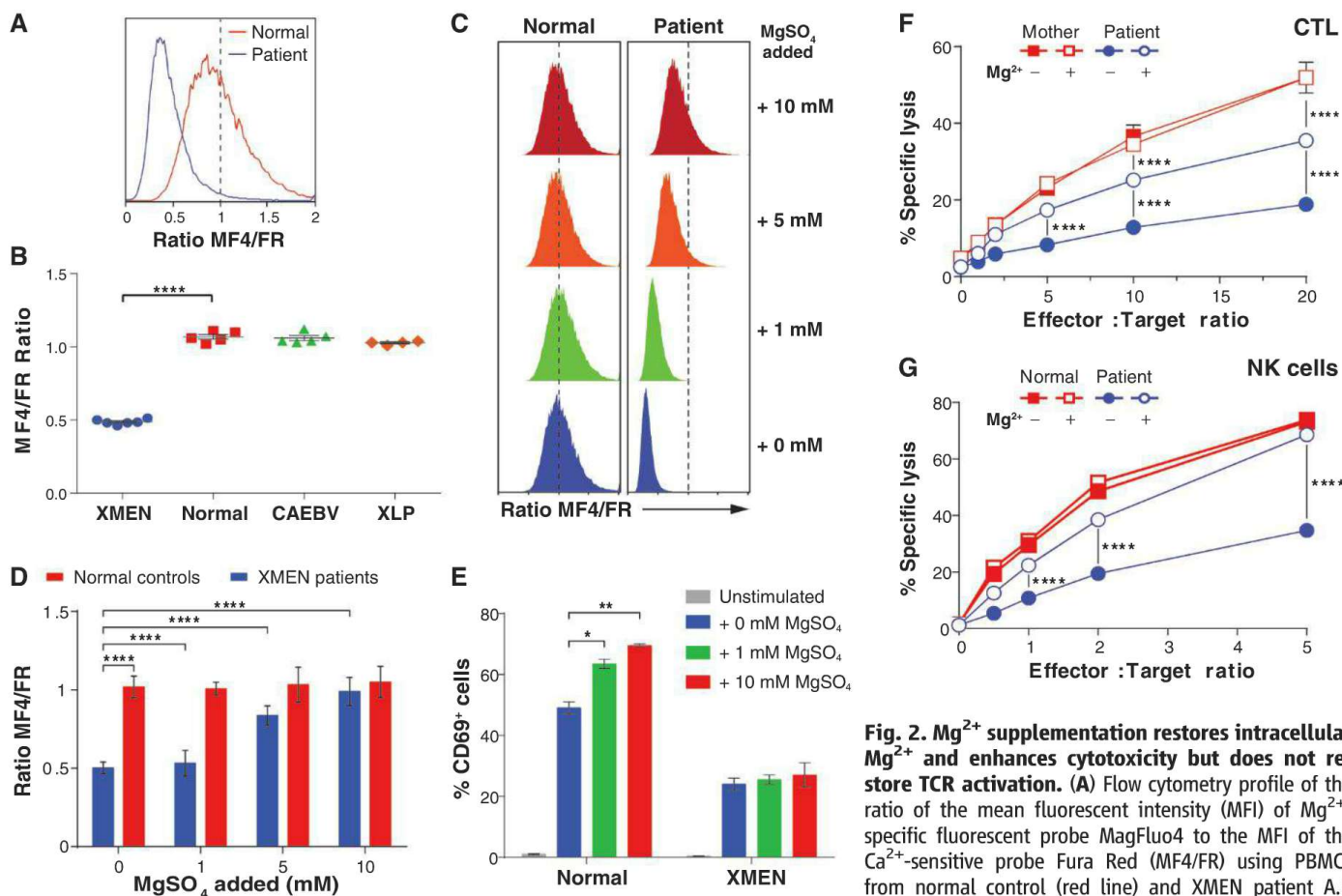


Fig. 2. Mg^{2+} supplementation restores intracellular Mg^{2+} and enhances cytotoxicity but does not restore TCR activation. (A) Flow cytometry profile of the ratio of the mean fluorescent intensity (MFI) of Mg^{2+} -specific fluorescent probe MagFluo4 to the MFI of the Ca^{2+} -sensitive probe Fura Red (MF4/FR) using PBMCs from normal control (red line) and XMEN patient A.1 (blue line). The ratio was arbitrarily set at 1 for normal control (dotted line). (B) Dot plot of the MF4/FR ratios from PBMCs of normal controls (squares, $n = 5$) and XMEN patients (circles, $n = 6$), non-XMEN CAEBV patients (triangles, $n = 5$), or XLP patients (diamonds, $n = 4$). (C) Flow cytometry profiles of MF4/FR ratio on normal control and XMEN patient CTLs cultured in media supplemented with the indicated amounts of $MgSO_4$ for 5 days. (D) MF4/FR ratio of CTLs from normal control (red bars, $n = 4$) or XMEN patients (blue bars, $n = 4$). (E) Percentage of $CD69^+$ in $CD8^+$ cells after stimulation with anti-CD3 (1 $\mu g/ml$) for 24 hours in the presence of the indicated amount of $MgSO_4$ or unstimulated. (F) Cytotoxicity of normal or XMEN patient EBV-specific CTLs, supplemented with or without 2 mM $MgSO_4$ for 5 days, on autologous LCLs. (G) Cytotoxicity of normal or XMEN patient NK cells, supplemented with or without 2 mM $MgSO_4$ for 5 days, on K562 targets. Results shown are mean $\pm$ SEM and are representative of all XMEN patients tested ($n = 4$) in at least three independent experiments (two-way ANOVA, $*P = 0.01$, $**P = 0.0038$, $****P < 0.0001$).

We therefore assessed NKG2D in XMEN NK cells and CTLs after 5 days of cultivation in medium with added MgSO_4 . We observed a rise in free $[\text{Mg}^{2+}]_i$ that correlated with increased NKG2D expression in both cell types (Fig. 3, F to G). The supplementation did not change the expression or function of other NK receptors or Mg^{2+} fluxes (figs. S4 and S8, A and B). Consistent with the lack of flux restoration, Mg^{2+} supplementation did not restore the CD16-redirected lysis by XMEN NK cells (fig. S8C). EBV⁺ LCLs expressed substantial levels of NKG2D ligands (fig. S9). The enhancement of CTL killing of autologous LCLs by Mg^{2+} supplementation in XMEN CTLs (Fig. 3H) appears to be NKG2D-specific because it was blocked by NKG2D-Fc (Fig. 3H). Thus, NKG2D expression is sensitive to free basal $[\text{Mg}^{2+}]_i$ and can be restored in XMEN patient cells by exogenous Mg^{2+} supplementation.

The defective killing by XMEN NK cells and CTLs associated with decreased free $[\text{Mg}^{2+}]_i$ might account for the failed immune control of

EBV. Therefore, we attempted to test this hypothesis in vivo by providing magnesium supplementation to patient A.1. First, we administered oral magnesium gluconate for 2 weeks. We observed a small but significant increase in basal free $[\text{Mg}^{2+}]_i$ and a modest restoration of NKG2D expression on both CTLs and NK cells (fig. S10). The fraction of B cells harboring intranuclear EBV-encoded small RNA (EBER) declined during treatment (fig. S10C). Twenty-one days after treatment cessation (day 36), we observed that basal free $[\text{Mg}^{2+}]_i$ dropped to the pretreatment level and the fraction of EBV-infected peripheral blood mononuclear cells (PBMCs) rebounded. These data indicate that continuous supplementation is required to maintain free $[\text{Mg}^{2+}]_i$ and a beneficial effect on EBV control. We also tested the infusion of magnesium sulfate for 3 days and observed stronger and faster responses (fig. S11). We then administered oral supplementation to patients A.1 and A.2 for 175 days with magnesium threonate (MgT), which has better bio-

availability and tolerability. Free $[\text{Mg}^{2+}]_i$ and NKG2D expression increased in both patients within 2 days of treatment (Fig. 4, A and B). The free $[\text{Mg}^{2+}]_i$ reached a plateau after 7 days of treatment and remained stable thereafter, whereas NKG2D expression increased progressively for the first 60 days (Fig. 4, A to C). The expression of NKG2D ligands observed on B cells was reduced in both patients (Fig. 4D). Moreover, the cells expressing NKG2D ligands were mostly EBV⁺ (Fig. 4, E and F). We noted a reversal of the fraction of EBV⁺ B cells in patient A.1 and to a lesser extent in patient A.2 at day 60 of treatment, which was later traced to a gap in Mg^{2+} administration. This indicates that the effects of Mg^{2+} supplementation were reversible and that control of EBV levels in vivo was linked to the level of NKG2D expression, which was continuously regulated by the free $[\text{Mg}^{2+}]_i$. The decrease in EBV-infected cells may be important because high levels of EBV are correlated with early lymphoma development and mortality in XMEN patients (table S1).

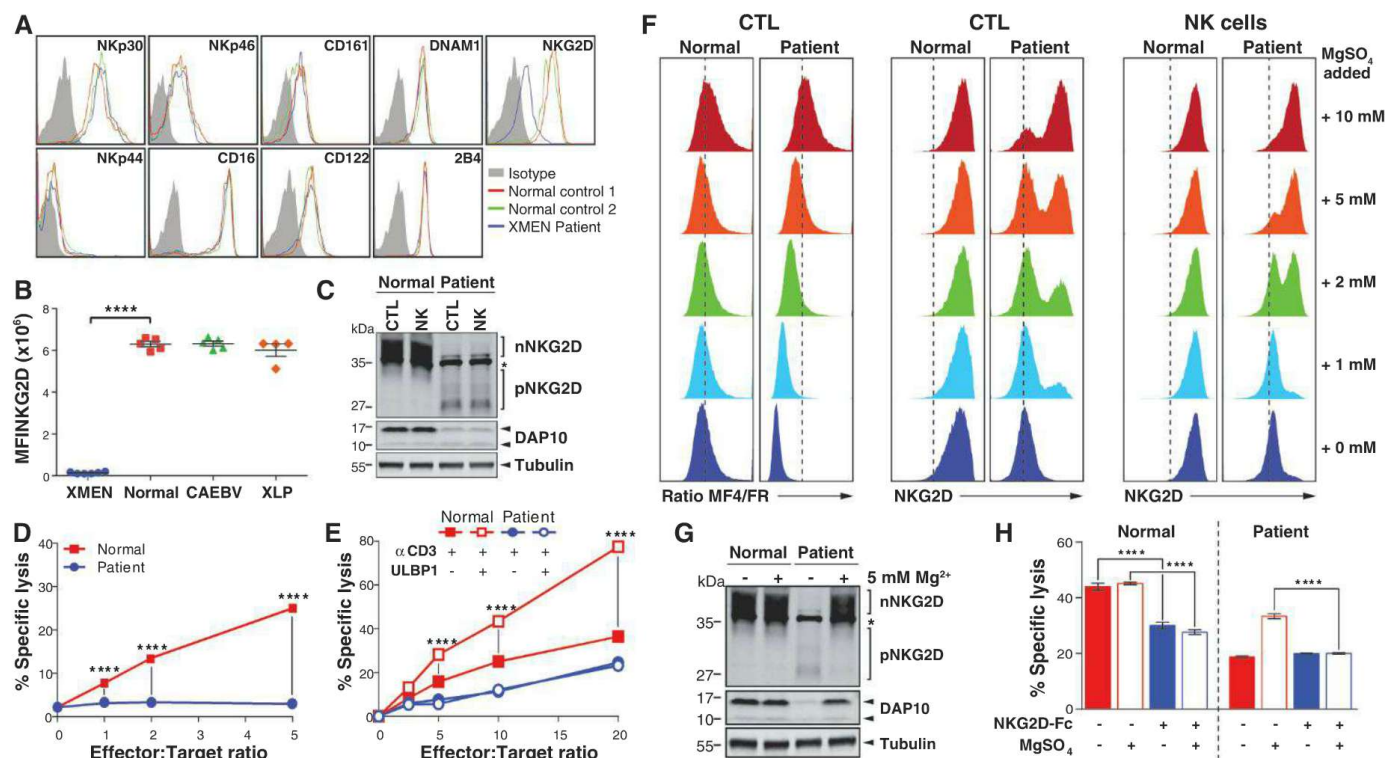


Fig. 3. Effect of Mg^{2+} on NKG2D expression and function in vitro. (A) Flow cytometry profiles of the surface expression of NK receptors on gated NK cells ($\text{CD}3^+ \text{CD}56^+$) from normal controls (red and green lines) and XMEN patient A.1 (blue line). Isotype antibody staining (gray shading) is shown as a reference for background fluorescence. Results are representative of all XMEN patients tested ($n = 5$). (B) Normalized MFI of NKG2D surface staining on gated CTLs from whole blood of normal controls (squares, $n = 5$) and XMEN patients (circles, $n = 6$), non-XMEN CAEBV patients (triangles, $n = 5$), or XLP patients (diamonds, $n = 4$). (C) Immunoblots of CTLs and NK cells from normal control and XMEN patient with the indicated antibodies. (D) NKG2D-specific redirected lysis of normal or XMEN patient IL-2-expanded NK cells on P815 cells expressing the NKG2D ligand, ULBP1. (E) NKG2D-specific redirected lysis of normal or XMEN patient CTLs on P815 expressing the NKG2D ligand ULBP1 or not in the presence of anti-CD3. (F) Flow cytom-

etry profiles of MF4/FR ratio in CTL (left) and NKG2D expression on CTLs (middle) and NK cells (right) from normal control or XMEN patient supplemented with the indicated concentrations of MgSO_4 for 5 days. (G) Immunoblot of cells lysates from CTLs from a normal control or an XMEN patient supplemented with or without 5 mM of MgSO_4 for 5 days. (H) Cytotoxicity of normal control or XMEN patient EBV-specific CTLs supplemented with or without 2 mM MgSO_4 for 5 days, on autologous EBV-transformed LCLs pretreated with or without soluble NKG2D-Fc. In (C) and (G), arrowheads show the various forms of the indicated protein. "nNKG2D" indicates the normal form of NKG2D and "pNKG2D" the patient's forms; an asterisk (*) indicates a nonspecific band detected by anti-NKG2D. Results shown are mean $\pm$ SEM and are representative of all XMEN patients tested ($n = 4$) in at least three independent experiments [Student's t test (B), two-way ANOVA [(D), (E), and (H)], **** $P < 0.0001$].

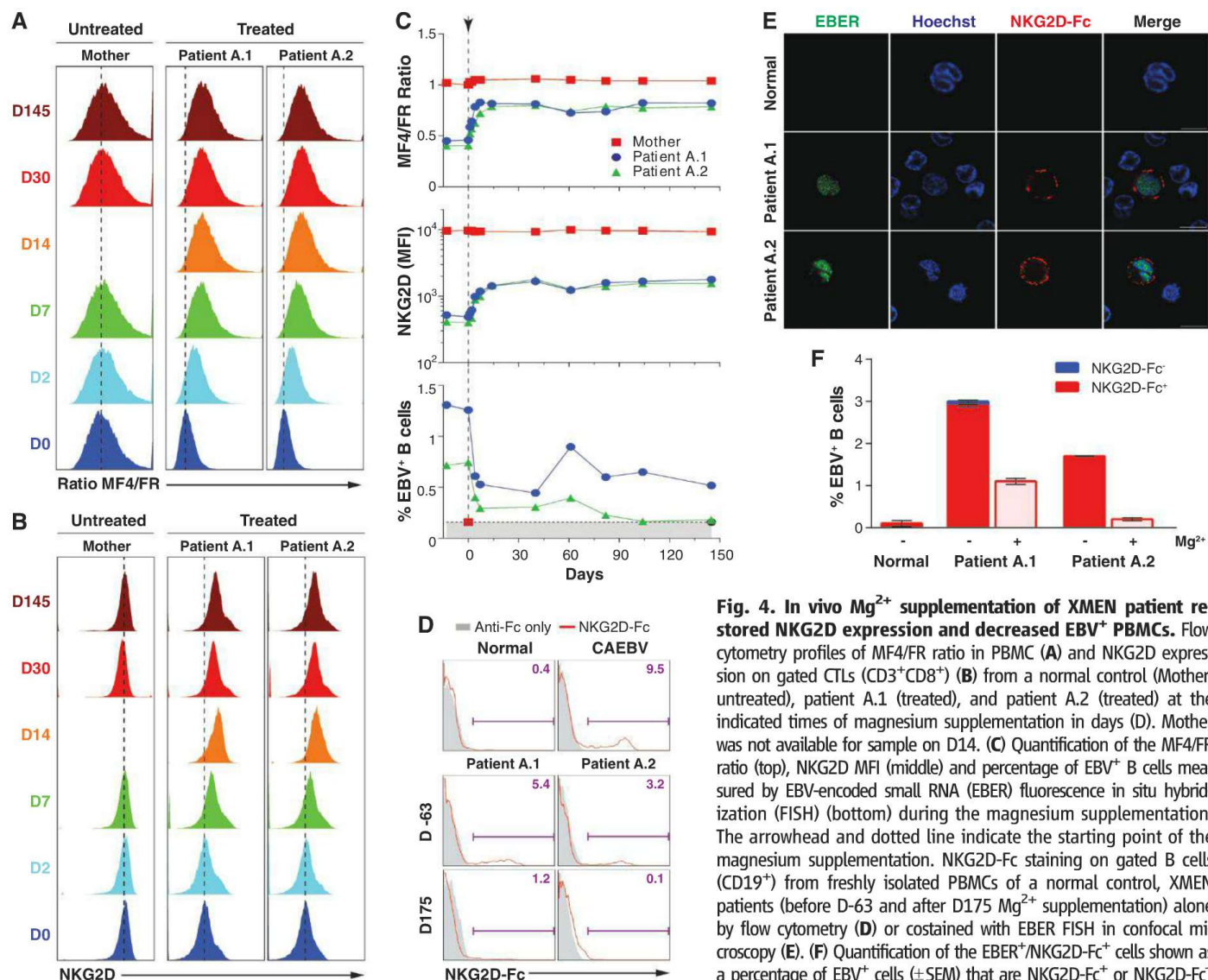


Fig. 4. In vivo Mg^{2+} supplementation of XMEN patient re-stored NKG2D expression and decreased EBV⁺ PBMCs. Flow cytometry profiles of MF4/FR ratio in PBMC (A) and NKG2D expression on gated CTLs (CD3⁺CD8⁺) (B) from a normal control (Mother, untreated), patient A.1 (treated), and patient A.2 (treated) at the indicated times of magnesium supplementation in days (D). Mother was not available for sample on D14. (C) Quantification of the MF4/FR ratio (top), NKG2D MFI (middle) and percentage of EBV⁺ B cells measured by EBV-encoded small RNA (EBER) fluorescence in situ hybridization (FISH) (bottom) during the magnesium supplementation. The arrowhead and dotted line indicate the starting point of the magnesium supplementation. NKG2D-Fc staining on gated B cells (CD19⁺) from freshly isolated PBMCs of a normal control, XMEN patients (before D-63 and after D175 Mg^{2+} supplementation) alone by flow cytometry (D) or costained with EBER FISH in confocal microscopy (E). (F) Quantification of the EBER⁺/NKG2D-Fc⁺ cells shown as a percentage of EBV⁺ cells ($\pm$ SEM) that are NKG2D-Fc⁺ or NKG2D-Fc⁻ in the patient's PBMCs during in vivo Mg^{2+} supplementation as measured by EBV-specific FISH. Scale bars, 5 μ m.

Our findings demonstrate that basal free $[Mg^{2+}]_i$ is regulated at least in part by extracellular Mg^{2+} and has concentration-dependent biological functions in eukaryotic cells besides the cofactor function of bound Mg^{2+} (1, 24, 25). The dynamic control of NKG2D expression by free $[Mg^{2+}]_i$ reveals the importance of $[Mg^{2+}]_i$ homeostasis for antiviral and antitumor immunity (Fig. 4C). There are more than 20 mammalian magnesium transporters, and it will be interesting to determine which control free $[Mg^{2+}]_i$ in XMEN patients during supplementation (5). XMEN patients manifest dysgammaglobulinemia, EBV infections, and susceptibility for lymphoma development similar to that exhibited by XLP patients (9, 26). In XLP1, 2B4- and NTB-A mediated cytotoxicity against EBV-infected B cells is defective (27–29). Given the loss of synergy between NKG2D and 2B4 in XMEN patients (figs. S6C and S13), 2B4-mediated recognition of EBV-infected cells may be impaired in XMEN

patients as well (23). Unlike cytomegalovirus, EBV does not have mechanisms to evade NKG2D recognition (30–32). NKG2D ligands are up-regulated by lytic induction of EBV-infected cells and in tumors from EBV⁺ posttransplant lymphoproliferative disorders (22, 33). Moreover, recent studies show that NKG2D plays a critical role in the success of antitumor and hematopoietic stem cell transplants (HSCTs) (34–36). Given that the only two known HSCT attempts in XMEN disease have been unsuccessful, Mg^{2+} supplementation may provide an adjunctive treatment in XMEN disease. Although the clinical utility of Mg^{2+} supplementation remains to be validated, our data illustrate in vivo that NKG2D is regulated by $[Mg^{2+}]_i$ and apparently plays a key role in EBV control.

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Acknowledgments: This research was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases and the National Cancer Institute, NIH. We thank H. Malech, A. Snow, J. Niemela, G. Fahle, and T. Dimaggio for assistance in various aspects of the research and patient evaluation and S. Holland for clinical insights and support. We also thank R. Germain, P. Schwartzberg, R. Siegel, T. Waldmann, K. Schafer-Weaver, C. Kanellopoulou, S. Muljo, D. McVicar, J. Milner, R. Baker, and S. Holland for critically reading the manuscript. We thank E. Long and S. Rajagopalan for sharing antibodies and cell lines and helpful assistance with NK experiments; we also thank K. Dowdell, R. Orentas, and B. Lafont for sharing reagents and D. Douek, D. Price, and

M. Quigley for assistance with tetramer staining. We are grateful to Merck Research Laboratories for generous support in kind of reagents and materials, and to P. R. Vagelos and R. Vessey for fostering the collaboration. F.-Y.L. was supported by the Medical Scientist Training Program at the University of California–San Francisco and the NIH M.D./Ph.D. Partnership program, and thanks K. Shannon and J. Toulmin for support and encouragement. We also acknowledge support by the Pharmacology Research Associate Training (PRAT) program (to C.L.L.), National Institute of General Medical Sciences, NIH. The data presented in this paper are tabulated in the main paper and in the supplementary materials.

Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6142/186/DC1

Materials and Methods

Author contributions

Figs. S1 to S13

Tables S1 and S2

References (38–44)

6 May 2013; accepted 10 June 2013

10.1126/science.1240094

Microcircuits for Hierarchical Elaboration of Object Coding Across Primate Temporal Areas

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In primates, neuronal representations of objects are processed hierarchically in occipitotemporal cortices. A “novel” feature of objects is thought to emerge and become prevalent at a cortical area because of processing in this area. We tested the possibility that a feature representation prevalent in a given area emerges in the microcircuit of a hierarchically prior area as a small number of prototypes and then becomes prevalent in the subsequent area. We recorded multiple single units in each of hierarchically sequential areas TE and 36 of macaque temporal cortex and found the predicted convergent microcircuit for object-object association in area TE. Associative codes were then built up over time in the microcircuit of area 36. These results suggest a computational principle underlying sequentially elaborated object representations.

Neuronal representations of objects are processed hierarchically in the primate occipitotemporal lobe (1, 2). Representations of a “novel” feature that constitutes a given object are thought to emerge and become prevalent in one of the areas in the cortical hierarchy (3, 4). Although this sudden emergence and prevalence of novel feature representations have been established for the transformation from a lateral geniculate nucleus to V1 in the visual processing pathway (5, 6), it is not known whether the same principle holds for the corticocortical hierarchy (7, 8) (see supplementary text for the background of the present study).

Previous studies have demonstrated that single neurons representing associations between two object images (pair-coding neurons) (9–11)

become prevalent in area 36 of the perirhinal cortex, whereas the pair-coding neurons only constitute a small minority in area TE (10, 11), a hierarchically prior cortical area (Fig. 1A) (12, 13). It is not known whether the activities of the small number of neurons in area TE merely reflect a possible variability of response selectivity or whether they emerge as a result of specific computations in a convergent microcircuit. Therefore, we looked for microcircuits that generated pair-coding neurons in areas TE and 36 by simultaneously recording from multiple single neurons in each of these areas (Fig. 1, B and C) while monkeys performed a pair-association memory task (fig. S1) (9–11, 14, 15). Conventional cross-correlation analyses were conducted to detect functional connectivity between recorded neurons (14–20) (see supplementary text for methodological issues on the functional connectivity). Figure 1, D to K, shows two example pairs of cells recorded in area TE. In both of these cell pairs, unit 1 exhibited a selective response to the optimal stimulus but did not respond to its paired associate (Fig. 1, F and J, top). Similar to unit 1, a selective response to the optimal stimulus was also

observed in unit 2 (Fig. 1, F and J, bottom). Unlike unit 1, however, unit 2 showed a substantial response to the paired associate, too. We then calculated cross-correlograms by using spikes elicited by the optimal stimulus to estimate the functional connectivity between units 1 and 2. We observed a prominent displaced peak on the right side of the shift-predictor-subtracted cross-correlogram (SSCC) (14, 15, 17, 18, 20), indicating the directed functional coupling from unit 1 to 2 in both cell pairs (Fig. 1, G and K).

In total, 70 pairs of regular-spiking putative excitatory pyramidal neurons (see materials and methods in the supplementary materials) were recorded from area TE of two monkeys, in which both of the constituent neurons in each pair exhibited stimulus-selective responses and at least one of the constituent neurons responded to both the optimal stimulus and its paired associate. The functional connectivity of each cell pair was then examined by calculating the SSCC for the responses to the optimal stimulus. A significant displaced peak on the SSCC was detected for 29 cell pairs based on the peak position and an asymmetry index (14–17, 20, 21), and the source and target units were determined for each pair on the basis of the coupling direction (fig. S2 and supplementary text). Source units represent leading neurons, and target units represent lagging neurons, in terms of relative spike timings on the SSCC (14, 22). As a population, both of the constituent neurons of these pairs showed prominent responses to their optimal stimuli [$P < 0.001$, versus baseline; one-way analysis of variance (ANOVA), followed by the least significant difference (LSD) test] (Fig. 2, A and B, fig. S3, and supplementary text). In contrast, responses to the paired associate of the optimal stimulus were observed only in the target units ($P < 0.001$) but not in the source units ($P > 0.08$), and the responses were significantly different between these units ($P < 0.02$, two-way ANOVA followed by LSD) (Fig. 2, A and C). These results support the view of functional convergence onto pair-coding neurons in area TE (Fig. 2G).

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The observed functional convergence was further supported by four different analyses. First, the majority of SSCCs with a displaced peak exhibited directed connectivity toward the neurons with stronger associative coding (22 of 29 cell pairs, 76%, $P < 0.006$, χ^2 test) (Fig. 2D), and the coupling strength was greater in the direction toward the neurons with stronger associative coding ($P < 0.03$, paired t test) (Fig. 2E, figs. S4 to S6, and supplementary text). Directed couplings toward neurons with stronger associative coding were observed

irrespective of whether target units responded more strongly to the optimal stimulus or its paired associate (13 and 9 of 22 pairs showing directed couplings toward neurons with stronger associative coding; $P > 0.3$, χ^2 test). Second, in addition to analyzing neuronal responses to the optimal pair of stimuli, we further evaluated the strength of associative coding by calculating a pair-coding index (PCI) (9–11), the response correlation for all the learned pairs of stimuli. The resultant PCI values were greater for the target units than for the source units ($P <$

0.02, paired t test) (Fig. 2F), and only the target units showed significant associative coding ($P < 0.006$). A third one on the Granger causality (figs. S9 to S11) and a fourth one on the connectivity dynamics (fig. S12) are detailed in the supplementary text.

We next examined the functional connectivity between neurons in area 36, which hierarchically locates at the next stage of area TE. In area 36, we recorded from 86 pairs of regular-spiking neurons in which both the constituent

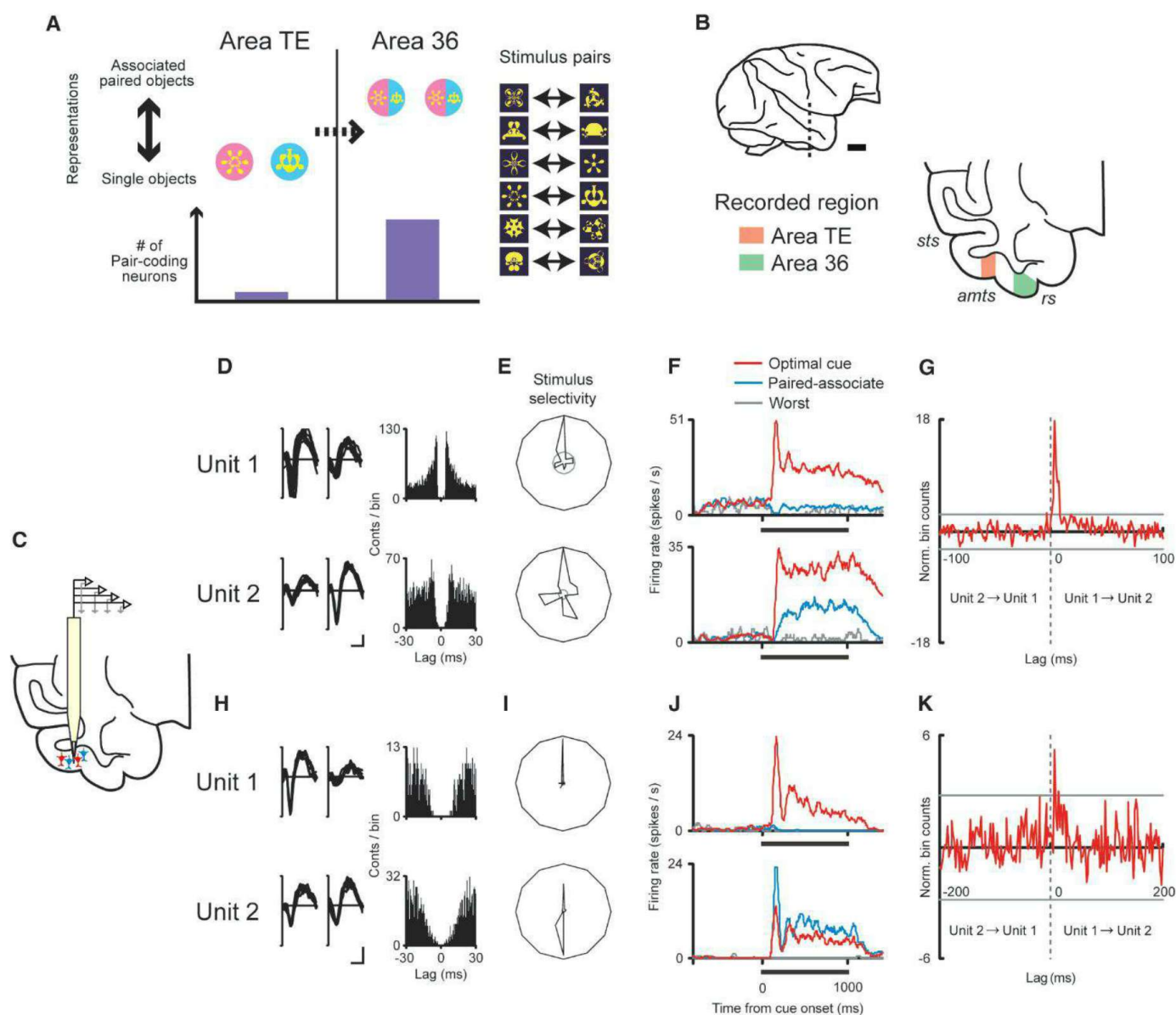


Fig. 1. Microcircuit analysis of object coding and example cell pairs. (A) (Left) Schematic diagram depicting hierarchical elaboration of object association across primate temporal areas. (Right) Stimulus set for monkey 1. **(B)** Lateral (top) and coronal (bottom) views of a monkey brain. Scale bar indicates 10 mm; dashed line, anteroposterior level of the coronal view; rs, rhinal sulcus; amts, anterior middle temporal sulcus; sts, superior temporal sulcus. **(C)** Simultaneous recordings of multiple single units. **(D to K)** Two example cell pairs and their functional connectivity. **(D and H)** Waveforms and autocorrelograms. Horizontal scale bars, 0.5 ms; vertical, 50 μ V. **(E and I)**

Stimulus selectivity of responses for each unit. Each vertex corresponds to each stimulus, and the vertices facing each other depict a pair of stimuli. Top and bottom vertices, optimal cue of the cell pair and its paired associate, respectively. Gray, baseline firing rate. Maximum, 29 and 23 spikes/s (100 to 600 ms from cue onset) for units 1 and 2 in (E) and 10 and 11 spikes/s for units 1 and 2 in (I). **(F and J)** Peristimulus time histograms (PSTHs) for the optimal stimulus and its paired associate. **(G and K)** SSCCs between units 1 and 2 for the optimal stimulus. Horizontal gray lines, confidence limit ($P < 0.05$, corrected for multiple comparisons).

neurons of each pair exhibited stimulus-selective responses, and at least one of the constituent neurons responded to both the optimal stimulus and its paired associate. Of these, 31 pairs of neurons exhibited a significant displaced peak on the SSCC in response to the optimal stimulus (fig. S13 and supplementary text). Both of the constituent neurons of these pairs showed prominent responses to their optimal stimuli ($P < 0.001$, one-way ANOVA followed by LSD) (Fig. 3A, fig. S14, and supplementary text) but not to their worst stimuli. In contrast to area TE, the paired associate of the optimal stimulus elicited responses from both of the constituent neurons in area 36 ($P < 0.02$ for source units, $P < 0.004$ for target units, two-way ANOVA followed by LSD) (Fig. 3B and fig. S14), without significant difference to each other ($P > 0.8$). SSCCs of area 36 neurons showed comparable probability of functional connectivity in directions toward neurons with stronger and weaker associative coding (55 and 45%, $P > 0.5$, χ^2 test) (Fig. 3C), and the coupling strength was indistinguishable between these directions ($P > 0.7$, paired t test) (Fig. 3D) (see figs.

S15 to S17 and supplementary text for further analyses). PCI analysis also revealed that both of the constituent neurons exhibited significant associative coding ($P < 0.01$ for source units, $P < 0.001$ for target units) (Fig. 3E) without difference to each other ($P > 0.6$). Applying a more stringent criterion for displaced SSCC peak replicated the above statistical results (fig. S18).

Because both the constituent neurons of a given pair exhibited associative representation in area 36, we next compared the dynamics of associative coding between these neurons. The dynamics of PCI values in the target units were aligned at the time when the PCI values of the corresponding source units reached the half maximum. In contrast to the rapid PCI increase in the source units, PCI values of the corresponding target units slowly increased after the peak in the source units (Fig. 3F). The response latency by itself was indistinguishable between these neurons ($P > 0.1$, paired t test). Whereas the PCI values of the source units decreased during the 300 ms after the half maximum ($P < 0.001$, paired t test) (Fig. 3G, left), PCI values of the corre-

sponding target units increased during the same period ($P < 0.02$) (Fig. 3G, right). As a result, the source and target units exhibited distinct PCI dynamics from each other ($P < 0.001$) (Fig. 3H). During this period, the target units in a significant majority of cell pairs exhibited a larger increase in the pair-coding index as compared with that of the source units (82%, 23 of 28, $P < 0.001$, χ^2 test) (Fig. 3I) (see supplementary text for discussion about the microcircuit operation in area 36 for object association).

The areal differences in the connectivity results were statistically supported by two two-way ANOVAs, each with a different dependent variable: Significant interactions were found both between factors of area (TE versus 36) and unit (source versus target) for the normalized response to the paired associate of the optimal stimulus ($P < 0.02$) and between factors of area and coupling direction (toward the neuron with stronger versus weaker associative coding) for the coupling strength ($P < 0.04$) (supplementary text). It should be noted that the observed areal differences in the connectivity results are not due to the relative

Fig. 2. Associative object representations through functional convergence in area TE. (A) Population PSTHs of constituent neurons of pairs with displaced SSCC peak [$n = 29$ for (A) to (F)] for the optimal stimulus and its paired associate. Thick and thin lines, mean and mean $\pm$ SEM. Horizontal black bars, cue period. A ± 25 -ms window was slid in 10-ms steps. (B) Population responses of constituent neurons of pairs with displaced SSCC peak for the optimal stimulus and its paired associate during the cue period. Error bars in all figures represent SEM. (C) Population responses for the paired associate, normalized to those for the optimal stimulus. (D) Proportions of cell pairs showing directed functional connectivity toward the neurons with stronger (left) and weaker (right) associative coding. (E) Population coupling strength in directions toward the neurons with stronger (left) and weaker (right) associative coding. (F) Population averages (left) and individual data (right) of pair-coding indices. ++, comparison with 0. (G) Schematic diagram depicting the observed functional convergence toward associative object representations in area TE. Note that the schema reflected asymmetric representation of object association in a TE neuron.

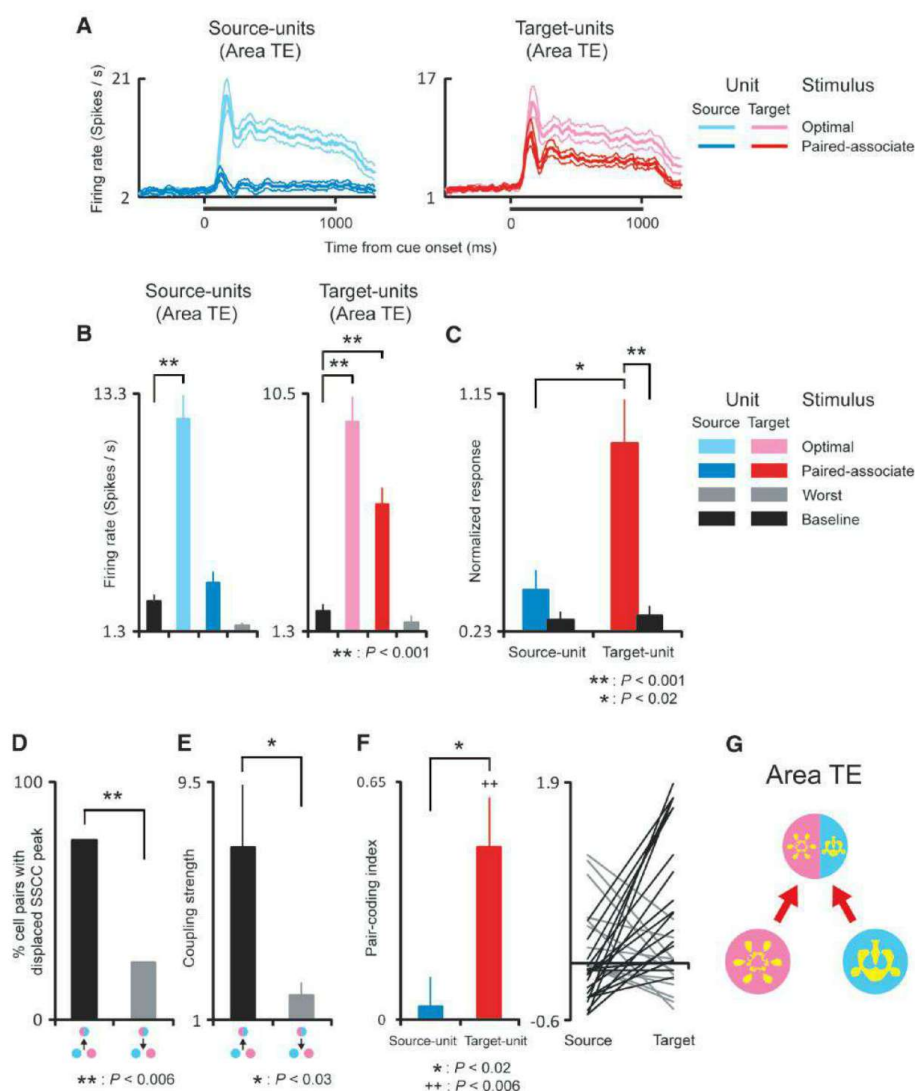


Fig. 3. Microcircuit for associative object representations in area 36.

(A) Population responses of constituent neurons of pairs with displaced SSCC peak [$n = 31$ for (A) to (E)] for the optimal stimulus and its paired associate during the cue period. (B) Population responses for the paired associate normalized to those for the optimal stimulus. (C) Proportions of cell pairs showing directed functional connectivity toward the neurons with stronger (left) and weaker (right) associative coding. (D) Population coupling strength toward the neurons with stronger (left) and weaker (right) associative coding. (E) Population averages (left) and individual data (right) of pair-coding indices. ++ and +, comparison with 0. (F to I) Dynamics of pair-coding indices in the constituent neurons of pairs with displaced SSCC peak ($n = 28$). (F) Population dynamics of pair-coding indices in the constituent neurons, aligned at the time of half-max value in the source units. Thick and thin lines, mean and mean $\pm$ SEM. A ± 100 -ms window was slid in 20-ms steps. (G) Individual data and (H) population average of the change in the pair-coding index during 300 ms after the half maximum in the source units. (I) Comparisons of the dynamics of the pair-coding index between the constituent neurons.

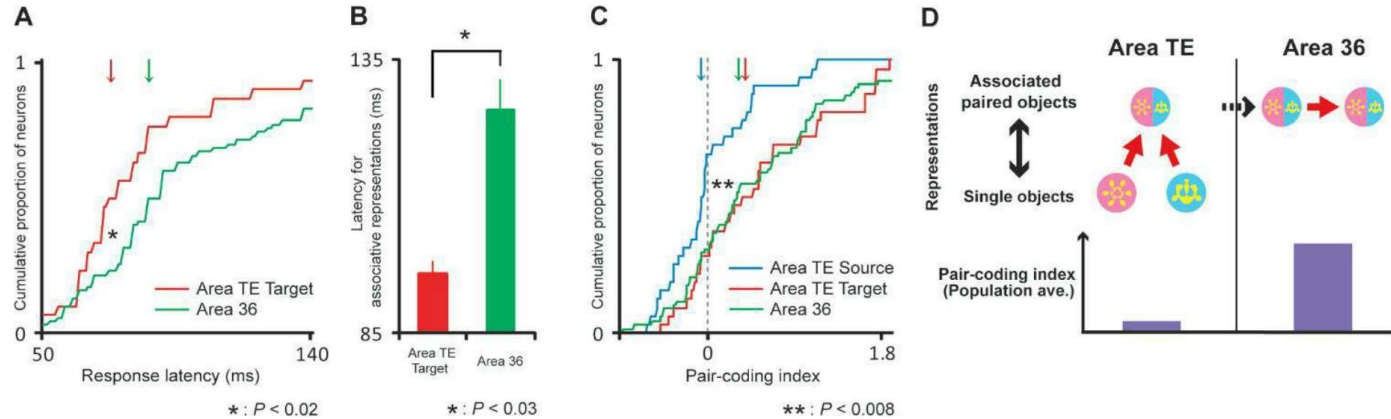
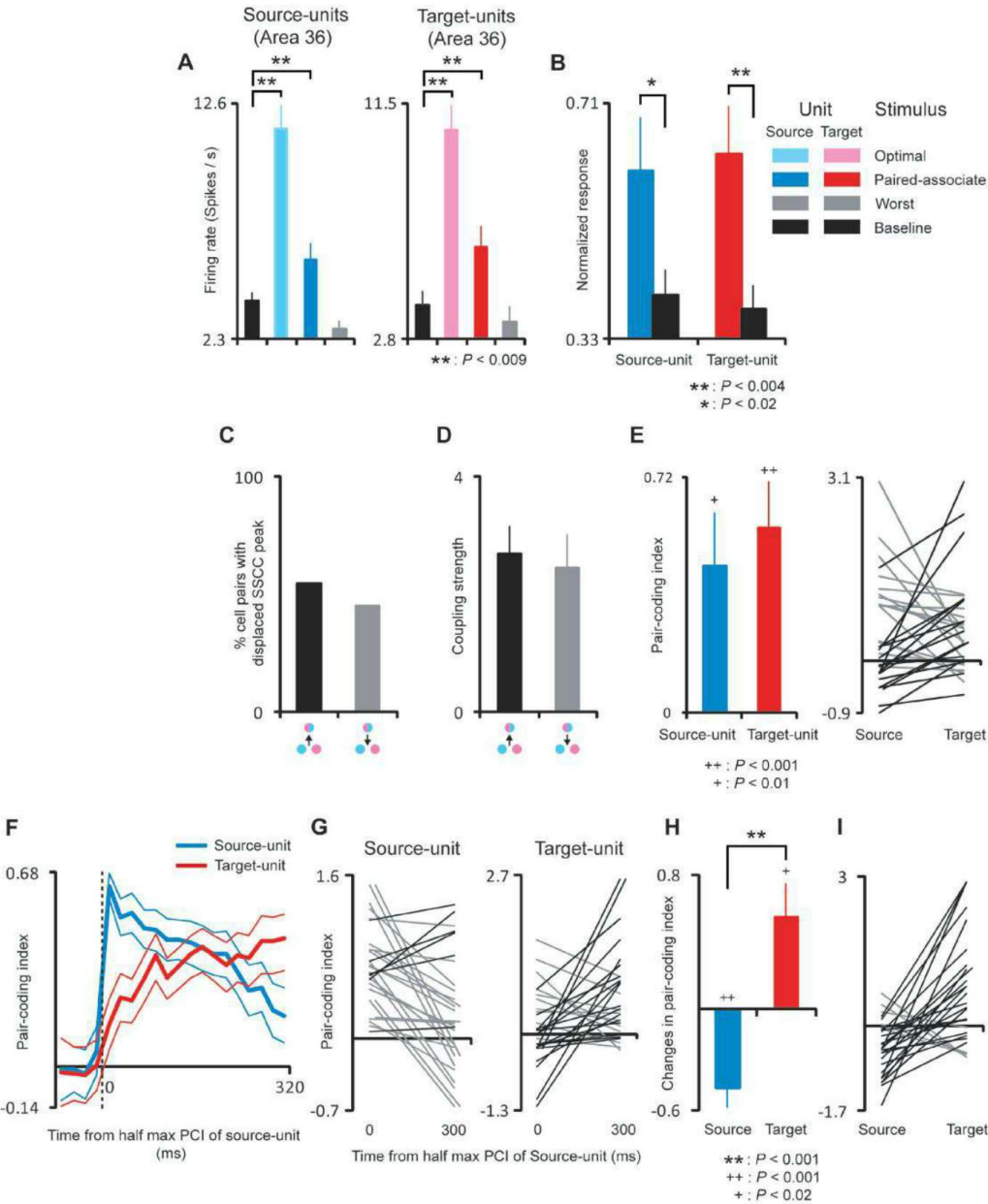


Fig. 4. Direct comparisons of response properties between target units in area TE and area 36 neurons. (A) Cumulative distributions of response latencies of target units in area TE ($n = 29$) and area 36 neurons ($n = 62$). Arrows, median values. (B) Population average latencies with which TE target units ($n = 29$) and

area 36 neurons ($n = 62$) represented the optimal pair of stimuli (i.e., responded to both stimuli). (C) Cumulative distributions of pair-coding indices for the source and target units in area TE ($n = 29$) and those for area 36 neurons ($n = 62$). Arrows, median values. (D) Schematic diagram depicting the results summary of the present study.

predominance of pair-coding neurons in area 36 (supplementary text).

In Fig. 4, response properties of target units in area TE were directly compared with those of area 36 neurons to characterize their hierarchical relationships.

First, the response latencies of the target units in area TE were significantly shorter than those of area 36 neurons ($P < 0.008$, Kolmogorov-Smirnov test) (Fig. 4A and figs. S20 to S21) (11, 23). Furthermore, the target units in area TE represented the optimal pair of stimuli with shorter latencies than area 36 neurons ($P < 0.03$, paired t test) (Fig. 4B), consistent with feedforward transfer of the associative representations from area TE to 36 (Fig. 4D).

Second, the PCI values also supported the hierarchical relations. As shown in previous studies, the PCI values were significantly greater in area 36 than in area TE, and significant associative representations were observed only in area 36 but not in area TE as a population (fig. S22) (10, 11). However, PCI values of the target units, but not the source units, in area TE were indistinguishable from those of area 36 neurons ($P < 0.008$ for TE source versus TE target, TE source versus area 36, Kolmogorov-Smirnov test; $P > 0.7$ for TE target versus area 36) (Fig. 4C), further supporting the notion that the prototype of associative representations can be generated in area TE as a result of microcircuit processing and then transferred to area 36, where the representations become prevalent (Fig. 4D).

Previous studies have attempted to uncover the functional hierarchy of cortical processing by showing the areal differences in the response properties of single units and presumed that novel feature representations emerge and become prevalent within a single cortical area (10, 11) (see supplementary text for the background of the present study). In the present study, we instead demonstrated the areal differences in the computational principles by detecting the functional microcircuits from different cortical areas and suggest

that the prevalence of elaborated object representations in a cortical area can be attained by emergence of the representations through the specific microcircuit computations in a prior area and subsequent building up over time in the microcircuit of the next area (see supplementary text for perspectives and limitations of the present microcircuit analyses). Because the present results were obtained with learned object associations, it remains elusive whether the present scheme can be applied for representations of more general object features (supplementary text).

The microcircuits for associative object representations found in the present study might act in parallel with other channels, including feedforward functional convergence from area TE (10, 11), top-down feedback from the prefrontal and/or medial temporal cortices (24–27), thalamic contributions (28, 29), and across-laminar interactions (14, 22) (supplementary text). Elucidating the entire view of across-areal elaboration of object representations will be an important issue for future studies to manifest the computational principles of the hierarchical brain.

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Acknowledgments: We thank G. Rangarajan for kindly providing the script to conduct the nonparametric Granger causality analysis, H. Kasahara and T. Watanabe for experimental assistance, and M. Takeda and K. W. Koyano for helpful discussions. This work was supported in part by Ministry of Education, Culture, Sports, Science, and Technology (MEXT)/JSPS KAKENHI grant nos. 19002010 and 24220008 to Y.M.; by CREST, Japan Science and Technology Agency to Y.M.; by a grant from Takeda Science Foundation to Y.M.; and a Grant-in-Aid for Young Scientists (B) from MEXT to T.H. (18700378).

Supplementary Materials

www.sciencemag.org/cgi/content/full/341/6142/191/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S23
References (30–87)

22 February 2013; accepted 11 June 2013
10.1126/science.1236927

Sample-Separation Technologies: Improving Speed and Resolution

Many scientific tasks—including studying the composition of blood and sequencing DNA—depend on being able to separate the parts from the whole. Only then can a scientist begin to understand how the pieces build up a process. To separate a sample into its components, scientists have been continually improving traditional approaches as well as developing new ones. In some cases, the improvements speed up separations; in others, advanced techniques provide new forms of separation that improve the efficiency of the processes. Though technologies are growing increasingly sophisticated, they are also becoming easier to use. **By Mike May**



Inside a Gas Chromatography Analyzer

In many ways, some of the greatest advances in separation science arise from how the technologies are applied. In metabolomics, for instance, researchers try to isolate all of the substances formed via an organism's metabolism. Studying the byproducts of these biochemical processes provides a challenge for scientists, and technology, since metabolites vary widely in their physical properties, such as size, charge, and concentration. To address these challenges, Vladimir Shulaev, professor of biological sciences at the **University of North Texas** in Denton, says, "We're involved in developing new applications for metabolomics and new analysis methods." For example, Shulaev and his colleagues explore ways to use advanced forms of liquid chromatography (LC) to screen metabolites.

Shulaev and other scientists rely on a collection of separation technologies, including LC and gas chromatography (GC), plus approaches that use features of both. The development of new separation techniques helps researchers learn more about basic biological systems by breaking them into parts. Separation technologies are also important for many

areas of applied science, such as drug discovery and development in the pharmaceutical industry.

TRENDS IN TECHNOLOGY

Although separation technologies include a broad collection of tools, industry representatives can identify some overall trends occurring across the board in the development of these techniques. As an example, Ed Horton, senior marketing manager for analytical products at **Beckman Coulter Life Sciences** (Indianapolis, Indiana), says, "The marketplace is driving toward more speed and [better] resolution. That's what seems to drive all of the techniques."

Another important trend that Horton mentions is that "separation technologies are starting to get more turnkey." That is, the technologies are becoming more application specific. For example, he points out that specific techniques are now available to separate complex sugars, intact proteins, peptides, and ions.

In LC, changes in the basic technology can speed up the process. According to Egidijus Machtejevas, global product manager for analytical chromatography at **Merck KGaA** (Darmstadt, Germany), "Monolithic technology is the future of chromatography." In traditional chromatography, samples move through columns packed with particles, but monolithic technology uses columns filled with a porous silica-gel rod, such as Merck's Chromolith High-Performance Liquid Chromatography (HPLC) columns. This monolithic approach can run four times faster than columns based on traditional particles. Also, Machtejevas points out that a scientist can easily upgrade to the monolithic approach by simply changing the column in an LC system.

Beyond changing columns, different forms of chromatography can be used to separate the various components of a sample. Some methods are better suited for isolating compounds with specific properties. For instance, hydrophilic interaction liquid chromatography (HILIC) does a good job of pulling out small polar compounds. "We have two HILIC products—ZIC-pHILIC polymeric columns and SeQuant ZIC-cHILIC columns—that can separate very challenging compounds," Machtejevas says. For example, the ZIC-cHILIC columns can be used to isolate melamine and cyanuric acid in infant formula.

Charged components in a sample can also be separated with ion-

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"The marketplace is driving toward more speed and [better] resolution. That's what seems to drive all of the techniques."

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"The major separation challenge is that these biological molecules are becoming more complex."

exchange chromatography (IC), which uses columns with charged sites to pull out ions. Linda Lopez, chromatography marketing manager at **Thermo Fisher Scientific** (Sunnyvale, California), says that IC is a good choice when "you need more information per unit time for closely eluting ions." She adds, "This works well for separating carbohydrates and glycans. You can do selective separations of highly branched carbohydrates." Researchers interested in metabolomics often study these molecules, especially glycans. Thermo Fisher Scientific's ICS-4000 and ICS-5000 are dedicated capillary IC systems. "These systems are reagent free," Lopez says. "You just add deionized water." Since the separations take place in capillary tubes, these systems don't even use much water—only about 5.25 L per year for the ICS-4000, according to product literature.

Many advances for separation technology focus on the stationary phase—the component in a column that captures the desired elements in a sample—but the mobile phase, or solvent, matters just as much. The solvent that carries the sample through the column plays an equally important role in the overall separation and including different additives in the solvent can also improve the separation. "The gold-standard additive for protein and amino-acid separations is trifluoroacetic acid (TFA)," says Tony Noonon, senior chemist at **CovaChem** (Loves Park, Illinois). If analyzing the separated analytes with mass spectrometry (MS), however, TFA can reduce the ionization efficiency of the sample, which effectively hides some of the peptides. To maintain the quality of the separation and allow sensitive analysis with MS, CovaChem offers a solvent-additive mixture that is 0.1 percent formic acid and 0.01 percent TFA. "This provides a good tradeoff between optimal peak separation and efficient analyte ionizations," Noonon says.

MOVING METHODS TO HPLC

Originally, the force of gravity pushed the mobile phase in LC, making it so-called low pressure LC. By using a pump to push the mobile phase, scientists developed HPLC. (Sometimes, HPLC is even described as "high-pressure" LC, instead of "high-performance" LC.) Typically, HPLC operates at pressures up to 5,000 pounds per square inch (psi). Ultra-HPLC (UHPLC) systems use pressures as high as 18,000 psi, or even higher.

Adding pressure to LC helps scientists separate compounds more completely. As Jens Trafkowski, product manager, analytical HPLC at **Agilent Technologies** (Santa Clara, California), says, UHPLC

provides "the ability to increase the efficiency and analytical power" of separations.

Some scientists run both HPLC and UHPLC. For example, a pharmaceutical company could already rely on validated assays for drug processing with HPLC that it doesn't want to change, but still want to explore new assays with UHPLC. Agilent developed its 1290 Infinity Quaternary LC System to run both methods. To run an HPLC method on UHPLC, Agilent developed its Intelligent System Emulation Technology (ISET). The ISET transfers the method from one platform to another, including both older Agilent LC platforms and even systems from competitors.

Other companies also develop versatile LC tools. For example, **Phenomenex** (Torrance, California), offers its Kinetex Core-Shell Technology LC columns. "These columns can be used on the newest UHPLC systems as well as older HPLCs that run at lower pressures," says Michael McGinley, product manager at Phenomenex. Late in 2012, Phenomenex added to the Kinetex line with a 5 μm column for small-scale preparative LC and a 1.3 μm column for use with UHPLC systems.

"We offer multiple particle sizes so that our columns are platform independent," McGinley explains. "Older instruments can still run the 5 μm and 2.6 μm columns."

NEXT GENERATION CHROMATOGRAPHY

Some people think that chromatography expands largely through incremental advances, but Richard Lee, chromatography marketing manager at **Bio-Rad** (Hercules, CA), believes that his company's next generation chromatography (NGC) brings a big change. The NGC systems are based on modular components with a plug-and-play format, and the modularity makes NGC very versatile. "A customer can buy whatever component they need—such as a pump to deliver buffers and samples, different detection systems, or buffer-blending—and add it to their NGC system to expand its capabilities," says Lee.

"Not all people who use chromatography are experts," Lee says. "So they may not be familiar with how to properly plumb a system or program a method." With the NGC system's ChromLab software, when a user clicks a flow path, LED lights flash on the hardware to show where plumbing connections must be made.

Advanced separation also benefits other techniques. **Sage Science** (Beverly, Massachusetts) develops separation technologies that prepare DNA for a variety of applications, including next generation sequencing. For many of these applications, says Chris Boles, chief scientific officer of Sage Science, "the size distribution has to be very tight." That means collecting only DNA that is within a few percent of the targeted length. The technology should also return as much of the targeted DNA as possible and have high reproducibility.

In most cases, a standard DC power supply drives the electrophoresis that separates DNA moving through an agarose gel. "Last year," says Boles, "we introduced an instrument, the BluePippin, that does pulsed-field electrophoresis. The traditional range of DC power lets you separate DNA samples as large as 10,000–15,000 bases, but our pulse-field approach expands that range up to 50,000 bases." He adds, "Pulsing the field slows down the electrophoretic *continued*>

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BIG OPPORTUNITIES WITH BIOPHARMACEUTICALS

Many of today’s drugs—including growth factors and interferons—are biological molecules. “The major separation challenge is that these biological molecules are becoming more complex,” says Laurens Sierkstra, chief executive officer at **BAC BV**, which was recently acquired by **Life Technologies** (Carlsbad, California). “Lots of biologicals have been pretty simple in the past, with simpler recombinant proteins and straightforward antibodies, but now there’s a totally new wave of molecules, including improved biologics with extended half-lives and antibody fragments.” He adds, “This makes everything more complicated from a separation point of view, and the process development is complicated and becoming even more [so].”

BAC’s CaptureSelect technology—an antibody-based approach to separation—can be used in large-scale manufacturing for purifying biologics. Life Technologies offers a range of products that purify antibodies, viruses, and other biologics. Sierkstra indicates that **Biogen-Idec**, a U.S.-based biotechnology company, used CaptureSelect technology in developing a purification strategy for its recombinant Factor VIII-Fc fusion molecule, and has submitted a biologics license application to the U.S. Food and Drug Administration. This biologic treats hemophilia A—an inherited defect that reduces blood’s ability to clot—and is a long acting recombinant Factor VIII. “Biogen wanted a non-animal-derived purification process,” says Sierkstra, “and we made this very simple by supplying to them an off-the-shelf purification product.”

Work in such biological drugs and biosimilars—biological pharmaceuticals that closely resemble innovative biotherapeutics—could drive increasing needs in separation technology. Both the original biologics and the biosimilars must be characterized during development. For such characterization, Horton says that many companies use Beckman

Coulter’s PA 800 *plus* Pharmaceutical Analysis System. This platform performs separations with sodium dodecyl sulfate (SDS)-based capillary electrophoresis. “This can be used to analyze samples for charge heterogeneity, molecular-weight heterogeneity, and other features,” Horton says.

This platform can also be used in other application areas, such as metabolomics. Urine, which often includes highly charged components, often serves as a sample for these studies. Because of that charge, says Horton, “you miss a large subset of the metabolites unless you use capillary electrophoresis.”

COMBINING TECHNOLOGIES

Separation often gets combined with detection. In fact, Hayley Crowe, global mass spectrometry commercialization leader at **PerkinElmer** (Waltham, Massachusetts), says, “In LC/MS or GC/MS, the instruments are being used in more labs as detectors.” She adds, “With that concept comes people who might not have much training in MS. So we have to come up with easier-to-use instruments that still give the quality MS results.”

To address this need, PerkinElmer developed the AxION iQT GC/MS/MS. This instrument includes a proprietary approach to MS that, Crowe says, provides a broad dynamic range that is similar to the triple-quadrupole MS, but has the accuracy and speed of a quadrupole time-of-flight MS instrument. To enable easy-to-run experiments, especially for inexperienced users, software handles much of the operation, including automatically optimizing the settings when working with known compounds. It is especially useful, Crowe says, “where someone needs to detect something that is at trace levels in a really dirty sample.”

To get even more information about the components in a sample, a scientist can use a different technology for separations. For example, the ACQUITY UPC² System from **Waters** (Milford, Massachusetts) provides convergence chromatography. Program Manager Mark Baynham explains that this system “uses compressed CO₂ gas and liquids as cosolvents.” With this combination of solvents, this technology can pull out components that might be overlapping in LC or GC separations. If a sample’s components are not clearly separated, the detector might miss them. “For instance if you’re looking for pharmaceutical impurities,” says Baynham, “you want to make sure that nothing is hiding, and [the ACQUITY UPC²] can help find things that other techniques miss.” He adds, “Neither the separation nor the detection is king; it is the optimization of both that leads to the best analytical answers, and we are working to make sure [our technology] is compatible with the full suite of mass spectrometers.”

Separating the many different components of a sample can require a range of tools, but in the end, the separation technique that a scientist chooses must also be optimized for the subsequent detection method, such as MS. Sample separations are often not meant to stand alone, but instead perform a crucial, early step in the scientific processes that underlie many new discoveries, ranging from deciphering an organism’s metabolome to finding key molecules for fighting cancer.

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DOI: 10.1126/science.opms.p1300078

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POSTDOCTORAL POSITIONS in Microbiology and Metabolomics

Postdoctoral positions are available for microbiologists and mass spectrometrists for enzyme and pathway discovery as part of the Enzyme Function Initiative (EFI). The EFI is a Glue Grant from NIGMS/NIH (U54GM093342) with the goal of developing sequence/structure-based strategies for facilitating assignment of in vitro enzymatic and in vivo metabolic functions of unknown enzymes discovered in genome projects, a crucial limitation in genomic biology. This is being accomplished by integrating bioinformatics, structural biology, and computation with enzymology, genetics, and metabolomics. Due to the collaborative and multidisciplinary environment, the EFI provides an opportunity to receive training in several areas. For example, those with a primary interest in microbial genetics can receive training in bioinformatics and/or in metabolomics. More information about the EFI can be found at [website: http://www.enzymefunction.org](http://www.enzymefunction.org) and to apply or request details, please contact **Professor John Cronan** (e-mail: jecronan@illinois.edu).

ASSISTANT PROFESSOR of Pollinator Health & Sustainability University of Maryland, College Park

The Department of Entomology seeks an Assistant Professor to develop an integrated Extension/Research/Teaching portfolio (40/40/20 split) that addresses the effects of environmental change on pollinators at multiple scales. The appointee will be expected to build a nationally prominent, robustly funded research/extension program that addresses some broad aspect of pollinator health and sustainability. In addition, he/she will work with university, government, and private sector/NGO partners to find novel ways of incorporating pollinators into multidisciplinary efforts that advance priorities of Maryland Extension, including, among others, agricultural sustainability, profitability, and literacy; environmental sustainability; local food production; and community resilience.

The appointee will also deliver innovative extension support and training in apiculture to diverse stakeholders in our state and region, train graduate students, and contribute to the department's teaching program. Applicants must have a Ph.D. in Entomology or a related field, demonstrated excellence in both scientific publication and ability to obtain external funding, and evidence of experience with Extension. Research specialties can include any aspect of pollinator biology that bears on pollinator health and sustainability. Preference will be given to candidates with postdoctoral experience, proficiency with Extension or outreach, and innovative approaches to translational science.

Electronic submission of application through the University online system ([website: http://www.ejobs.umd.edu](http://www.ejobs.umd.edu)) is required. Candidates should submit a cover letter describing qualifications, curriculum vitae, a summary of research and extension experience and future plans (three to four pages), a statement of educational interests, and contact information for three persons from whom letters of recommendation can be requested. When preparing the electronic submission, submit the statement of research and extension in the Research field, and the statement of educational interests in the Supplemental Documents field. The position remains open until filled. Best consideration will be given to applications received by August 1, 2013.

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Contact the Admissions Office, **telephone: 800-824-5526** at The New England College of Optometry, 424 Beacon Street, Boston, MA 02115. Additional information, **website: <http://www.neco.edu>**; e-mail: admissions@neco.edu.

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The American Association for the Advancement of Science (AAAS) is a non-profit organization dedicated to serving society through the advancement of science. The *Science* Research Journals are seeking a lively, detail-oriented **Editorial Coordinator** to work with a small team of editors in our Washington DC office. This is an excellent opportunity for a computer-comfortable individual interested in scholarly publishing to gain broad exposure to and experience in many aspects of publishing.

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- Extensive university or college level training leading to a Bachelor's degree in a relevant field
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- Excellent written and oral communication skills including interaction with scientific authors
- Strong computer skills, particularly in Word and Excel
- Familiarity with copyediting is a plus

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UNIVERSITY of MISSOURI



ENDOWED DISTINGUISHED FACULTY SCHOLAR POSITION – DIABETES RESEARCH

The Department of Internal Medicine and Division of Endocrinology at the University of Missouri invites applications for appointment as the Thomas W. Burns MD Distinguished Faculty Scholar, an endowed tenured or tenure-track position (Assistant, Associate, or Full Professor; rank commensurate with experience) for clinician-scientists whose research and clinical interests focus on diabetes. The successful candidate is anticipated to lead his/her own independent extramurally-funded research program and to work with Dr. James Sowers and others in integrating basic and clinical translational research within the School of Medicine and other academic units on campus. The successful candidate will be able to devote 70% effort to research.

This recruitment effort is part of a multi-year expansion for the department and is designed to complement existing areas of research strength in diabetes, obesity, vascular biology, cellular signaling, and/or translational diabetes research. The University is noted for its collaborative research programs, which are exemplified by multidisciplinary interactions that are facilitated by appointments within the Burns Center for Diabetes and Cardiovascular Research, the Dalton Cardiovascular Research Center, the College of Veterinary Medicine, the Life Sciences Center, and the National Center for Gender Physiology. Involvement in campus-wide research initiatives relative to diabetes and obesity, cardiovascular science, cell signaling, proteomics, membrane biology, nutrition, genetics, or gender physiology is desirable.

Please submit your curriculum vitae online at <http://hrs.missouri.edu/find-a-job/academic/> and forward a narrative of research interests, and the names and contact information of three references to:

Chair, Burns Faculty Scholar Search Committee
Department of Internal Medicine
School of Medicine
University of Missouri
One Hospital Drive
Columbia, MO 65212

Or by electronic submission (strongly preferred) to:
BurnsScholar@health.missouri.edu

Active review of applications will begin July 1 and the search will continue until the position is filled.

The University of Missouri is an equal access, equal opportunity, affirmative action employer that is fully committed to achieving a diverse faculty and staff. For more information, call the Associate Vice Chancellor of Human Resource Services/Affirmative Action officer at 573-882-4256. To request ADA accommodations, please call Human Resource Services at 573-882-7976. TTY users, please call through Relay Missouri, 1-800-RELAY (735-2966) or en Español at 1-800-520-7309.

Visit the University of Missouri-Columbia's web site at <http://mujobs.missouri.edu>

Faculty Position in Cardiovascular Science

The Virginia Tech Carilion Research Institute (VTCRI) in Roanoke, Virginia (<http://research.vtc.vt.edu/>) is recruiting a faculty member working in the area of cardiovascular function in health and disease. The position may be filled at the Assistant or Associate Professor level. The successful candidate will have a Ph.D. (or M.D./Ph.D. or D.V.M./Ph.D.) and postdoctoral training experience. Investigators with strong innovative research programs and extramural funding are encouraged to apply although promising junior candidates will be considered. Start-up packages, facilities and support are highly competitive. The VTCRI opened in the summer of 2010 under the leadership of Dr. Michael Friedlander. It currently has 22 research teams with \$50M in startup funding and an additional \$45M in current active extramural research funding. Under the leadership of Dr. Robert Gourdie, the VTCRI recently established the Center for Heart and Regenerative Medicine Research (CHRM). The Center and Institute have state of the art facilities in optical, high field electron and magnetic resonance imaging, as well as molecular biology, electrophysiology, computation and high capacity data analysis/storage facilities. During this period of major growth of the new institute, we are especially interested in colleagues who enjoy a highly collaborative environment and interacting with investigators from their own as well as other disciplines including those working at molecular, cellular, whole organismal, bioengineering and computational levels with cellular and/or animal models and/or humans. Investigators using physiological, molecular genetic and/or imaging approaches to study cardiovascular function are encouraged to apply. The VTCRI is immediately adjacent to the Carilion Clinic and the new VTC School of Medicine where all medical students carry out 4 year research projects. The Institute has strong collaborative ties with the Virginia Bioinformatics Institute and the School of Biomedical Engineering and Sciences at Virginia Tech (VT) as well as with the VT Departments of Biological Sciences, Biochemistry, Physics, Psychology and the College of Veterinary Medicine. The research institute and medical school are located in the picturesque Roanoke Valley midway between Washington DC and Charlotte, NC. Send cover letter, statement of research interests and CV and have at least three referees send letters of support to Ms. Sarah Castle, HR Director at secastle@vtc.vt.edu by August 18, 2013. Indicate cardiovascular position on all correspondence.

Virginia Tech has a strong commitment to the principle of diversity, and in that spirit seeks a broad spectrum of candidates including women, minorities, veterans, and people with disabilities.

Faculty Position in Cancer Biology

The Virginia Tech Carilion Research Institute (VTCRI) in Roanoke, Virginia (<http://research.vtc.vt.edu/>) is recruiting a faculty member working in the area of cancer biology. The position may be filled at the associate or full professor level. Start-up packages, facilities and support are highly competitive. The successful candidate will have the opportunity to take a leadership role in the growth of the cancer biology research program at the VTCRI, including the recruitment of several additional faculty. The VTCRI opened in the summer of 2010 under the leadership of Dr. Michael Friedlander. It currently has 22 research teams with \$50M in startup funding and an additional \$45M in current active extramural research funding. The Institute has state of the art facilities in optical, high field electron and magnetic resonance imaging, as well as cell and molecular biology, structural biology, nanotechnology, developmental biology, biophysics, computation and high capacity data analysis/storage facilities. During this period of major growth of the new institute, we are especially interested in colleagues who enjoy a highly collaborative environment and interacting with investigators from their own as well as other disciplines including those working at molecular, cellular, whole organismal and computational levels. The successful candidate will have a Ph.D. (or M.D./Ph.D. or D.V.M./Ph.D.), postdoctoral training experience and a successful track record of running an innovative extramurally funded cancer research program. The VTCRI is immediately adjacent to the Carilion Clinic and the new VTC School of Medicine where all medical students carry out 4 year research projects. The Institute has strong collaborative ties with the Virginia Bioinformatics Institute, the Virginia Tech (VT) Center for Drug Discovery and School of Biomedical Engineering and Sciences as well as with the VT Departments of Biological Sciences, Biochemistry, Human Nutrition, Physics, Psychology and the College of Veterinary Medicine. The research institute and medical school are located in the picturesque Roanoke Valley midway between Washington DC and Charlotte, NC. Send cover letter, statement of research interests and CV and have at least three referees send letters of support to Ms. Sarah Castle, HR Director at secastle@vtc.vt.edu by August 18, 2013. Indicate cancer position on all correspondence.

Virginia Tech has a strong commitment to the principle of diversity, and in that spirit seeks a broad spectrum of candidates including women, minorities, veterans, and people with disabilities.

PURDUE UNIVERSITY

Senior Faculty Position in Experimental Particle Physics Department of Physics

The Department of Physics at Purdue University seeks applications for a tenured faculty position at the Associate or Full Professor rank in the area of experimental particle physics. We are interested in outstanding scientists with an established track record, international stature, a commitment to leading a preeminent research program, and a clear vision for future development.

Purdue has major responsibilities in the CMS experiment and is involved in the Mu2e, XENON100/XENON1T, STAR, LSST and VERITAS projects. Synergies exist with groups in astrophysics, theory, nuclear physics and condensed matter physics. The department offers a state-of-the-art in-house facility with resources applicable to silicon detector design, development and fabrication.

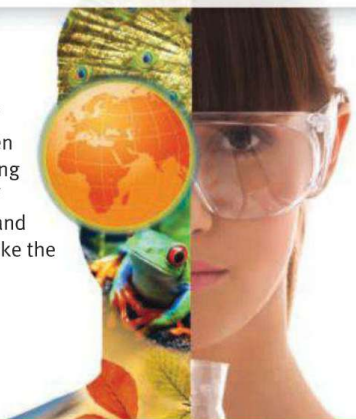
It is expected that the successful candidate will strengthen the current efforts and play a leading role in shaping the future of the group. Applicants must have a Ph.D. in physics or a related field, an outstanding record of research accomplishments, and evidence of excellence in teaching at both the undergraduate and graduate levels. Candidates are expected to supervise graduate students, teach undergraduate and graduate courses, and serve on university committees. Salary and benefits are highly competitive.

Questions regarding this position and search should be directed to the Chair of the search committee, **Professor John Finley** (finley@purdue.edu). Interested candidates should submit their curriculum vitae, a list of publications to which the applicant was instrumental, brief descriptions of their planned research program and of their teaching philosophy, as well as names and email addresses of four people from which the search committee can obtain letters of reference. Electronic submission at <https://www.physics.purdue.edu/searches/app/> is preferred. Review of applications will begin **October 1, 2013** and will continue until the position is filled. A background check is required for employment in this position. Purdue University is an ADVANCE institution.

Purdue University is an Equal Opportunity/Equal Access/Affirmative Action Employer fully committed to achieving a diverse workforce.

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The Ohio State University

Assistant Professor of Evolution, Ecology and Organismal Biology

The Ohio State University at Lima seeks candidates for a full-time, tenure-track Assistant Professor position in Evolutionary Biology. A research specialty in Comparative Vertebrate Biology is preferred, but not required.

The appointment will be made in the Department of Evolution, Ecology, and Organismal Biology at The Ohio State University and begin in August 2014. Candidates must have a PhD in hand at the time of appointment; post-graduate teaching experience is preferred. Salary is competitive.

The department seeks an Evolutionary Biologist with a strong commitment to the teaching, research and outreach missions of The Ohio State University. The successful candidate will teach undergraduate surveys and major courses in their area of expertise (likely courses include introductory biology for honors students, vertebrate biology, and comparative vertebrate physiology). In addition, the position will require the candidate to pursue a distinguished record of service and scholarly research.

The Ohio State University at Lima is one of five campuses of The Ohio State University. Current enrollment on the Lima campus is 1,400 students and there are approximately 100 full- and part-time faculty in all academic departments. Ohio State Lima offers the first two years of the Ohio State general education curriculum and ten programs leading to baccalaureate degrees, including one in Biology. Ohio State Lima also offers Master's degree programs in Education and Social Work.

Review of applications will begin on September 16, 2013, and will continue until the position is filled. Please send a cover letter, a current curriculum vita, and three letters of recommendation to:

Dr. Eric Juterbock, Chair, EEOB Search, c/o Whitney Clark, Office of Human Resources, Public Service Bldg. 222, The Ohio State University at Lima, 4240 Campus Drive, Lima, OH 45804.

Questions should be addressed to Dr. Eric Juterbock at Juterbock.1@osu.edu.



To build a diverse workforce Ohio State encourages applications from individuals with disabilities, minorities, veterans, and women. EEO/AA employer. For additional information about the Lima campus please see www.lima.osu.edu



CHEMISTRY
TEXAS A&M UNIVERSITY

Head of Chemistry

Applications and nominations are invited for the position of Head of the Department of Chemistry at Texas A&M University. The ideal candidate should have an internationally recognized research program, a demonstrated commitment to undergraduate and graduate education, and administrative experience.

The Department of Chemistry has 58 full-time faculty members, 270 graduate students, 65 postdoctoral associates and 250 undergraduate majors (www.chem.tamu.edu). The department houses energetic and diverse research programs in all areas of chemistry and has excellent instrument facilities. Texas A&M University, the 5th largest university in the country, has more than 50,000 students and a multi-billion dollar endowment. The College Station/Bryan area is consistently ranked among the best places to live in the country.

Applicants should e-mail a detailed curriculum vitae to: headsearch@chem.tamu.edu. Review of applications will begin **October 1, 2013** and continue until the position is filled.

The Texas A&M University System is an Affirmative Action/Equal Opportunity Employer (www.tamus.edu/offices/eo). The university is dedicated to building a culturally diverse and pluralistic faculty and staff committed to teaching and working in a multicultural environment; applications from women, minorities, individuals with disabilities, and covered veterans are strongly encouraged. Texas A&M University is aware that attracting and retaining exceptional faculty often depends on meeting the needs of two careers and having policies that contribute to work life balance. For more information, visit <http://dof.tamu.edu/content/balancing-work-and-life>. Applicants must have a doctoral degree in chemistry or a related field.

Max Planck Institute of Immunobiology and Epigenetics

Max-Planck-Institut für Immunbiologie und Epigenetik



We are looking for a

Postdoctoral Position in Bioinformatics

The Max Planck Institute of Immunobiology and Epigenetics in Freiburg, Germany is offering a Postdoctoral Position in Bioinformatics in the Laboratory of Chromatin Regulation (Head: Dr. Asifa Akhtar). The position is available for an initial two-year appointment with the possibility of extension.

The MPI in Freiburg is an international research institute at the cross-road of Southern Germany, Switzerland and France. The working language is English. State-of-the-art infrastructure and service units, including transgenesis, mass spectrometry, proteomics, flow cytometry, fly and imaging facilities are available.

Your tasks:

The research focus of the Akhtar laboratory includes mechanisms underlying chromatin and epigenetic regulation. We are particularly interested in X chromosomal regulation using flies and mouse models employing multidisciplinary approaches such as genetics, biochemistry, functional genomics as well as cell biology and structural biology.

We are looking for enthusiastic, highly-motivated, science-driven and experienced postdoctoral fellows to join our team to unravel the molecular mechanisms that regulate gene expression.

Your qualifications: Applicants should have a PhD or equivalent doctoral degree with at least 3 years of proven research experience in bioinformatics and analyses of genome-wide data (ChIPseq, RNA seq). Prior experience working in *Drosophila* or mammalian models is highly encouraged. Candidates must have a strong publication record. Furthermore, the ability to work in a team, communication skills and experience in the supervision of graduate students are an asset.

Please submit your application, including a statement of research interests, a CV and three references to our homepage: <http://www.ie-freiburg.mpg.de/jobs>

We offer: Salaries will be according to postdoctoral fellowships of the Max Planck Society or TVöD and will commensurate with experience.

Application deadline: 31.08.2013

Our institute investigates the molecular basis of the immune response and other topics of the developmental biology, such as the origin and differentiation of the immune cells as well as the development of vertebrate embryo. Another main focus of the institute is Epigenetic. This area deals with inheritable traits, which are not caused by changes in the DNA sequence.

Handicapped applicants with equal qualifications will be given preferential treatment. The Max Planck Society seeks to increase the number of women in areas, where they are underrepresented, and therefore explicitly encourages women to apply. A childcare facility is directly attached to the institute.

If you would like to work in a dedicated team, please convince us now by sending us your complete application documents together with your salary expectations and your earliest possible date of joining the institute.

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DR. SHIRLEY MALCOM



To Dr. Shirley Malcom, born and raised in the segregated South more than 65 years ago, a career based on her studies in science seemed even less likely than the launch of the Soviet's Sputnik. But with Sputnik's success, the Space Race officially started and, in an instant, brought a laser-like focus to science education and ways to deliver a proper response. Not long after, Dr. Malcom entered the picture.

Although black schools at the time received fewer dollars per student and did not have sufficient resources to maintain their labs at a level equivalent to the white schools, Dr. Malcom found her way to the University of Washington where she succeeded in obtaining a B.S. in spite of the difficulties of being an African American woman in the field of science. From there she went on to earn a Ph.D. in ecology from Penn State and held a faculty position at the University of North Carolina, Wilmington.

Dr. Malcom has served at the AAAS in multiple capacities, and is presently Head of the Directorate for Education and Human Resources Programs. Nominated by President Clinton to the National Science Board, she also held a position on his Committee of Advisors on Science and Technology. She is currently a member of the Caltech Board of Trustees, a Regent of Morgan State University, and co-chair of the Gender Advisory Board of the UN Commission on Science and Technology for Development. She has held numerous other positions of distinction and is the principal author of *The Double Bind: The Price of Being a Minority Woman in Science*.

Of her active career in science, Dr. Malcom says, "I guess I have become a poster child for taking one's science background and using that in many other ways: we ask questions; we try to understand what we find; we consider what evidence we would need to confirm or refute hypotheses. And that happens in whatever setting one finds oneself."

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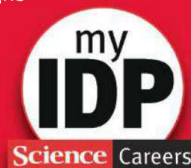
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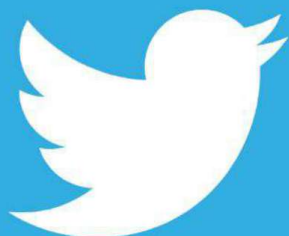
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Research Scientist

The Division of Urologic Surgery at Brigham and Women's Hospital and Harvard Medical School invites applications for Research Scientist position at the level of Assistant or Associate Professor. The Division seeks candidates with expertise and track-record of peer review funding in cell and molecular biology in areas related to urologic diseases. The position includes opportunities for leadership in the development of program projects with the institution and mentoring of residents, fellows and young faculty. Strong collaborative environment with Dana-Farber Cancer Institute and Harvard School of Public Health.

Candidates should send curriculum vitae, preprints/reprints of significant work (limit to 3), statement of present and future goals in investigation and arrange to have three letters of recommendation sent to **Adam S. Kibel, M.D., Chief, Division of Urology, Brigham and Women's Hospital, Department of Surgery, 75 Francis Street, Boston, MA 02115** or email to lcates@partners.org. Other inquiries call (617)-732-6665.

Brigham and Women's Hospital and Harvard Medical School are Equal Opportunity/Affirmative Action Employers. Women and minorities are encouraged to apply



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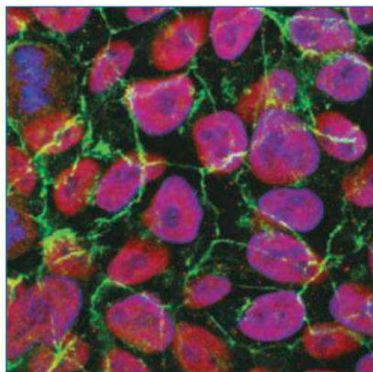
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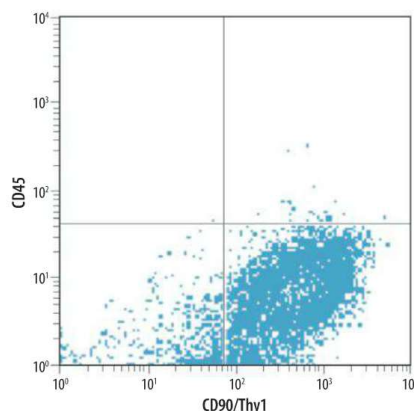
More characterization With less variation

The Broadest Selection of Stem Cell Antibodies

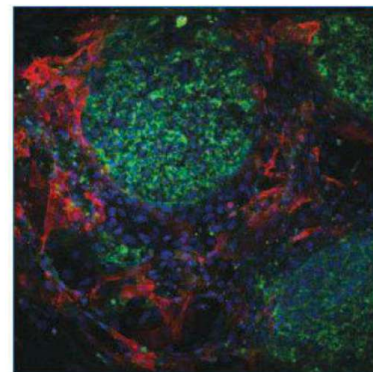
- ✓ From established stem cell markers to newly identified molecules
- ✓ Manufactured & qualified in-house
- ✓ Optimized for immunocytochemistry & flow cytometry



Oct-4A (red) & E-Cadherin (green)
in iPS2 human induced pluripotent stem cells.



CD90/Thy1 & CD45
in human mesenchymal stem cells.



SSEA-1 (red) & SSEA-4 (green) in live iPS2
human induced pluripotent stem cells.

**Increase the Probability of
Experimental Consistency & Success**

www.RnDSystems.com/StemCellAbs

R&D Systems manufactures over 12,000 high performance antibodies to facilitate thorough characterization of stem cell potency status and the signal transduction mechanisms that regulate differentiation and proliferation. We also offer GloLIVE™ azide-free antibodies to confirm marker expression in live unfixed cells and Proteome Profiler™ antibody arrays to simultaneously detect 15 stem cell markers.